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THE UNIVERSITY OF ALBERTA

SYNAPTIC TRANSMISSION IN THE SIXTH

PERIPLANETA AMERICANA L.

by

© Peter Kai Leung Chiang

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ENTOMOLOGY

EDMONTON, ALBERTA

AUGUST 1967

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    The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled Synaptic Transmission in the Roach, Periplaneta americana (L.) submitted by Peter Kai Leung Chiang in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Fluctuations in the endogenous activity and synaptic transmission were observed in intact, in situ nerve cords of P. americana treated with insect saline and Mazola corn oil. Both the endogenous activity and synaptic transmission were either partially or entirely blocked by 10^{-3} M hemicholinium, and after 10^{-3} M choline was applied, the endogenous activity and synaptic transmission returned to normal. Carbachol (10^{-2} M) inhibited electrical activity of the nerve cords entirely.

Nicotine (10^{-3} M) stimulated immediately upon application, followed by an irreversible block. Dimethylphenylpiperazinium (10^{-2} M) and methacholine (10^{-2} M) produced no observable effect. Pilocarpine (10^{-3} M) and Tremorine (10^{-2} M) depressed the endogenous activity and synaptic transmission. Acetylcholine at 10^{-3} M produced no observable effect, but at 10^{-2} M exhibited a progressive blocking effect without apparent stimulation. Eserinized nerve cords were washed with saline and acetylcholine was applied immediately, but no stimulation was observed. Choline (10^{-2} M) blocked synaptic transmission in four out of six preparations.

Eserine (10^{-5} M) caused facilitation and synaptic block one hour after application. Sevin and Zectran (10^{-3} M) produced immediate stimulation followed by a period of complete electrical quiescence. Prolonged repetitive discharge alternated with synaptic block. Eventual electrical block followed. Tetraethyl pyrophosphate (10^{-4} M)

produced similar effects. After treatment with TEPP (10^{-3}, 10^{-4} M), the synaptic transmission and endogenous activity of four nerve cords was reactivated by pyridine-2-aldoxime methiodide (10^{-3} M). Choline (10^{-3} M) apparently did not reactivate the electrical activity of TEPP-treated nerve cords. Diazinon (10^{-2} M) produced the same effects as other anticholinesterases.

Assayed colorimetrically, the AChE activity of individual cercal ganglia varied considerably. The mean AChE activity of TEPP-treated ganglia was 11.16% of the control.

Phenoxybenzamine and tranylcypromine (10^{-3} M) blocked both the endogenous activity and synaptic transmission of the nerve cords. Fluorometric assay did not reveal the presence of dopamine or noradrenaline in the abdominal nerve cords.

The results suggest that the synaptic transmission of roach cercal ganglion is dependent upon ACh and AChE.

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I.   INTRODUCTION

Adrian (1930) reported that isolated nerve cord of a caterpillar
exhibited electrical discharge, which was abolished when the ganglia were
destroyed. He also noted electrical discharges from the isolated nerve
cord of Dytiscus sp. (Adrian, 1931). After the nerve cord had been in
saline an hour or longer, the general discharge in many fibers tended to
decline, leaving activity that consisted of steady trains of impulses in
the residual fibers and periodic bursts having a frequency of 5 to 15
impulses per minute. Adrian (1941) suggested that these bursts of activ-
ity corresponded with the breathing rhythm of the intact insect and
represented activity of respiratory centers. In the isolated abdominal
nerve cord of Melanoplus sp. such periodic bursts did not occur (Roeder,
1953). Smalley (1963) observed that ventral nerve cord of Blaberus
craniifer (Burmeister) generated rhythmic bursts of impulses which cor-
responded to the expiratory movements of the intact roaches. Since much
of the electrical activity in the isolated nerve cord appeared unrelated
to external stimulation, Roeder (1939, 1953) first termed it "spontaneous
activity," and later "endogenous activity" (Roeder, Tozian and Weiant,
1960; Roeder, 1963).

Endogenous activity may provide a background of nerve impulses
for the maintenance of some degree of muscle tonus, under conditions
approaching zero sensory input (Roeder, 1953; Hoyle, 1966), and res-
piratory control (Smalley, 1963). Endogenous activity forms a basis of
insect behavior (Hoyle, 1966; Roeder, 1962, 1963; Wigglesworth, 1965).

Insect neurons do not differ greatly from those of other animals in basic physiology (Adrian, 1930; Roeder, 1953; Treherne, 1966). Fundamentally, the membrane resting and action potentials of insects are the same as those described by the ionic theory advanced by Hodgkin and Huxley (Narahashi, 1963, 1964). According to this theory, the nerve of insects in the resting state is more permeable to potassium than to sodium ions. The potassium concentration is much higher in the axoplasm than the external medium or haemolymph. The situation is opposite in respect to sodium. Action potential production can be explained as the transient increase in membrane conductance to sodium and potassium. The spike potential is followed by an undershoot or positive phase which is terminated in a negative afterpotential, explicable by the accumulation of potassium released during activity in the immediate vicinity of the nerve membrane.

Skou (1964, 1965) discussed the enzymatic basis for active transport of Na^+ and K^+ across cell membranes. A ($Na^+ + K^+$)-activated enzyme system has been isolated from crab peripheral nerves and rat brain (Ahmed and Judah, 1965b; Skou, 1964, 1965). There are indications that this enzyme system is involved in the active transport of Na^+ and K^+ across the cell membrane. Ahmed and Judah (1965) suggested that phosphoserine of alkaline phosphatase was the active center of the enzyme in brain lipoprotein.

Sodium and potassium have a profound influence on the resting and action potential of insects (Hoyle, 1952; Yamasaki and Narahashi, 1959b; Narahashi, 1963). A decrease in the sodium concentration has an

appreciable effect on the resting potential; the rate of rise of the action potential was decreased very effectively by the sodium-deficient solution. An increase in potassium concentration caused depolarization. Results of radioisotope experiments showed that the exchanges of inorganic ions and molecules occur relatively rapidly between the haemolymph and the central nervous system in both the roach, Periplaneta americana (L.) (Treherne, 1961a,b; 1962b,c) and the stick insect, Carausius morosus (Br.) (Treherne, 1965a). In the roach, the high levels of cations and low concentration of chloride ions in the extracellular fluid is due to a Donnan equilibrium with the haemolymph (Treherne, 1966). Sodium ions are extruded from the roach nerve cord by a secretory mechanism which is associated with the uptake of potassium ions (Treherne, 1961a,b,c; 1965a). The rate of entry of $^{42}K^+$ into the nerve cord was fourteen times that of $^{24}Na^+$ (Treherne, 1961a).

Hagiwara and Watanake (1956) demonstrated that the resting potential of cicada motor neurons was 60 mV. and the peak voltage of its action potential was 75 mV. The giant axon of Periplaneta americana had a resting potential of 77 mV. and an action potential of 99 mV. (Narahashi, 1963).

Communication among neurons is achieved by the process of signal transmission at specialized regions of neuronal contact, the synapses. A synapse is a junction between nerve cells (Eccles, 1965; Patton, 1965). At the synapse, the presynaptic terminal has vesicles which contain a special organic substance, called transmitter substance (Eccles, 1965). When a nerve impulse reaches the presynaptic terminal,

some of the vesicles eject the transmitter substance into the synaptic cleft. The molecules of substance diffuse across the cleft in only a few microseconds and become attached to specific receptor sites on the surface on the postsynaptic membrane. During the attachment, sodium and potassium ions flow through the membrane of the postsynaptic nerve thousands of times more readily than during the rest phase, producing an intense ionic flux that depolarizes the cell membrane and produces an excitatory postsynaptic potential (Eccles, 1964, 1965). The transmitter substance is then eliminated from the synaptic cleft, either by diffusion into the surrounding regions or as a result of being destroyed by enzymes.

There are three main types of neurons in the insect nervous system (Wigglesworth, 1965): the sensory, motor, and association neurons. The sensory neurons are generally bipolar; a distal process, or dendrite, runs to a sense organ adapted to receive some particular type of stimulus, and a proximal process, or axon, runs to the central nervous system. The cell bodies of the motor neurons are situated in the central nervous system. They are unipolar, devoid of dendrites, and located in the peripheral part of the ganglion where they divide into a collateral and an axon filament. The collaterals are connected with those of association neurons of sensory neurons. The axon filaments constitute the motor nerves. The association neurons or internuncials are classified as segmental or multisegmental according to whether they react solely to input into one ganglion or into a number of consecutive ganglia. The internuncials are further subdivided into ascending interneurons belonging

to the giant fiber system, and neurons restricted to the protocerebrum which form the mushroom bodies.

Insect neurons are enclosed by Schwann cells or glial cells (Wigglesworth, 1965). The glial cells invaginate the plasma membrane of the neuron to form a "mesaxon," or suspensory fold, which may be wrapped a few times around the neuron. The space between the layers of glial cells is called the "glial lacunar system" (Wigglesworth, 1960). In the ganglion, the glial cells become specialized. One type is called "perineurium cells" (Wigglesworth, 1965). These cells have abundant mitochondria and store glycogen (Wigglesworth, 1965). The "neural lamella" consists of a neutral mucopolysaccharide containing collagen fibrils arranged in layers with different orientations (Wigglesworth, 1958a).

In P. americana, a system of multisegmental neurons has been described which is called the giant fiber system (Hess, 1958; Roeder, 1948a, 1953, 1962). Afferent fibers from mechanoreceptors on the caudal segments converge on the last abdominal (cercal) ganglion and synapse with a small number of giant fibers. The giant axons ascend the nerve cord through the abdominal ganglia, and there appears to be no synapse at any point in the giant fibers of the abdominal nerve cord (Hess, 1958; Roeder, 1948a). Some of the giant fibers apparently ascend to the brain without interruption, and others synapse in the thoracic ganglia with motor neurons supplying the leg muscles (Hess, 1958). The giant fiber system mediates evasive response of the insects (Roeder, 1948a, 1962). In the intact roach, puffs of air applied to the sensillae

on the cerci induce an alarm reaction causing evasive response (Roeder, 1948a, 1962).

In vertebrates, acetylcholine plays a vital role in preganglionic transmission of the autonomic nervous system, skeletal neuromuscular junctions, and in central nervous system (Koelle, 1963, 1965; and others). Acetylcholine may also have a role in adrenergic transmission (Burn and Rand, 1965; Koelle, 1963). Acetylcholine (ACh) and the enzymes, choline acetylase (ChA) and acetylcholinesterase (AChE) which facilitate ACh synthesis and hydrolysis respectively, have been demonstrated in the insect tissues including nervous tissue, yet the physiological role of ACh in insects remains to be demonstrated (Colhoun, 1963b; Smith, 1965).

Acetylcholine has been suggested as a transmitter substance in the cockroach nervous system (Smith and Treherne, 1965; Treherne, 1966; Yamasaki and Narahashi, 1960). Gautrelet (1938) demonstrated the presence of ACh in bees. Conteggian and Serfaty (1939) reported ACh in eight species of insects. An increase of ACh content was demonstrated in nerve cords of P. americana treated with eserine (Colhoun, 1958b; Mikalonis and Brown, 1941). Roach CNS contains 40-200 µgm ACh/gm. of nerve cord (Mikalonis and Brown, 1941). Similar values for ACh content of the roach nerve cord were also given by other investigators (Lewis and Smallman, 1956; Tobias, Kollros and Savit, 1946). ACh content in the sixth abdominal ganglion was 63 µgm./gm. (Colhoun, 1958a), and is located in "structural compartments." Cholineacetylase, the enzyme for the synthesis of ACh, was demonstrated in blowfly heads (Smallman, 1956).

True acetylcholinesterases have been isolated from the CNS of

Acheta domesticus (L.) (Edward and Gomez, 1966). The molecular weight of a fly head AChE was estimated by gel filtration and sucrose density gradient to be 160,000 (Drysan and Chadwick, 1966).

Investigating synaptic and axonic transmission in P. americana, Roeder (1948b) first concluded that there was no evidence showing that ACh was a synaptic transmitter of nerve activity, although anticholin-esterase disrupted synaptic transmission in the sixth abdominal ganglion. The conclusion was also based on the failure of high external concentra-tions of ACh, other choline esters, and cholinergic blocking agents to interfere with synaptic conduction.

Later, Roeder and Kennedy (1955) proposed that organophosphates could, besides inhibiting AChE, block ACh receptors at high concentrations. To circumvent the ineffectiveness of externally applied ACh, Twarog and Roeder (1956, 1957) desheathed the last abdominal ganglion, and observed that ACh, between 10^{-3} M. and 10^{-2} M., exerted a rapid and pronounced effect on the synaptic transmission of the ganglion. In two of a series of seventeen experiments, only a moderate decrease in synaptic response was noted. In all others, within one to five minutes, bursts of syn-chronous action potentials were followed by synaptic depression and block. Desheathing also reduced the concentrations at which other pharmacological agents affect synaptic transmissions. This led to the postulation of an ion barrier theory, which held that the connective tissue sheath investing the roach nervous system retards the passage of ions from the bathing medium to the interior of ganglion and connectives (Twarog and Roeder, 1956, 1957). But the authors also pointed out the possibility of well

protected synapses in insect ganglia (Roeder and Twarog, 1957).

Based on his observations on the electrical activity of Locusta migratoria (L.), Hoyle (1952, 1953) also suggested that the sheath surrounding peripheral nerves and ganglia of insects may act as a selectively permeable barrier, separating the molecules and ions of the haemolymph from those of the nervous system. O'Brien (1957, 1959a,b) reported that ionized pharmacological compounds had low toxicity to insects. Histological studies demonstrated that methylthiocholine was completely prevented from penetrating the intact nerve sheath (Winton, Metcalf and Fukuto, 1958).

Treherne (1961a,b,c; 1962a,b), and Treherne and Smith (1965a, 1965b) demonstrated that inorganic ions and ACh do penetrate the nerve tissue of insects. The influx of ^{14}C-labelled ACh into the extracellular system of the roach nerve cord occurred extremely rapidly, with a half-time of approximately 50 seconds (Treherne and Smith, 1965a). The insect nervous system is not virtually isolated beneath an impermeable nerve sheath, but is in a dynamic equilibrium with some smaller ions and molecules in the haemolymph (Treherne, 1965b).

Using the light microscope, intense AChE activity was demonstrated in the neuropile, sheaths encapsulating the neuron perikarya, and the perineurium of the nerve sheath of the insect CNS (Iyatomi and Kaneshina, 1958; Wigglesworth, 1958b). Electron microscopy has revealed the following distribution of eserine-specific esterase activity in the 6th abdominal ganglion of the roach, P. americana (Smith and Treherne, 1965): (1) in the glial sheaths of the axons in the connectives and

cercal nerves; (2) in the glial folds encapsulating the neuron peri-
karya in the ganglion; (3) in localized areas along the membrane of
axon branches within the neuropile, frequently in association with local
clusters of synaptic vesicles.

The controversy about the exact function of ACh as a transmitter
substance was further enhanced by the conclusions of Pringle and Hughes
(1948), and O'Connor, O'Brien and Salpeter (1965) that the neuromuscular
junction of insects is not cholinergic.

Another choline, β,β-dimethyl acrylcholine, was found in the
prothoracic gland of the garden tiger moth Arctia caja (Bisset et al.,
1960). But Chang and Kearns (1955), and Colhoun and Spencer (1959) failed
to demonstrate the presence of any free choline esters, other than ACh in
the nervous system of the cockroach. Colhoun (1963) pointed out the
difficulty in accepting ACh as a synaptic transmitter first because of
the failure to prove that ACh accumulated in nervous tissue as a free
ester following stimulation in the presence of anticholinesterases, and
second, the lack of evidence for antidromic stimulation or the blocking
effects of some pharmacological agents, such as curare and atropine.

Statement of the Problem

It was my purpose to carry out a systematic study of:

(1) the nature of the synapse of the 6th abdominal ganglion of the roach, P. americana, by using various pharmacological agents;

(2) the function of ACh and AChE in the synapse;

(3) whether catecholamines are present in the synapse.

II. EXPERIMENTAL METHODS

1. Introduction:

Pumphrey and Rawdon-Smith (1937), and Roeder (1948a) have shown that stimulation of the cercal sensillae of P. americana elicits a volley of impulses from the cercal sensory fibers which pass to the sixth abdominal ganglion, and the impulses subsequently pass to the nerve cord. Colhoun (1958b, 1960) studied synaptic transmission in the 6th abdominal ganglion in situ by air puffing onto the cerci. Adaptation to air puff was not shown in the 6th abdominal ganglion (Colhoun, 1960). There was an increase in total ACh content in eserinized, isolated nerve cords, which were stimulated by air puffing (Colhoun, 1960). But there was no detectable change in total ACh content in the in situ nerve cords and the 6th abdominal ganglia, treated the same way (Colhoun, 1960).

Since each step involved in neurohumoral transmission represents a potential point of drug attack, Koelle (1965) proposed the following four possible approaches for identifying a cholinergic synapse by considering the prototype drugs that affect processes concerned in each step:

(1) Interference with the release of the transmitter:

Hemicholinium (HC-3) can block synaptic transmission by blocking the transport system by which choline accumulates in the terminals of cholinergic fibers, and thus, it limits the synthesis of ACh.

(2) Promotion of the transmitter release:

Carbachol is supposed to act by releasing ACh at the synapse. It also probably acts directly at postsynaptic cholinergic receptors.

(3) Combination with postsynaptic receptor sites:

When a drug combines with a receptor, two effects may be observed: the same effect as that of ACh (i.e., cholinomimetic); or no apparent direct effect but, by occupying the receptor site, the drug prevents the action of endogenous ACh (i.e., cholinergic blockade).

(4) Interference with the destruction or dissipation of the transmitter:

The primary action of anticholinesterases, such as organophosphates and carbamates, is the inhibition of AChE, with the consequent accumulation and action of endogenous ACh at sites of cholinergic transmission. All drugs in this class probably have in addition, direct actions at cholinoreceptive sites, and elsewhere.

2. Electrophysiological Studies:

2.1 Materials:

Acetylcholine chloride, Diazinon (0,0-Diethyl 0-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate), Nicotine, Sevin (N-Methyl-1-naphthyl carbamate), and Zectran (4-Dimethylamine-3,5-xylyl methylcarbamate) were obtained from City Chemical Corp. DMPP (1,1-Dimethyl-4-phenyl piperazinium) was supplied by Dr. Graham Chen of Parke, Davis & Company. Acetyl-beta-methyl choline chloride, eserine sulfate, and Tremorine (1,4-Dipyrrolidine-2-butyne) were obtained from Nutritional Biochemicals Corp. TEPP (Tetraethyl pyrophosphate) was supplied by California Chemical Co. Choline chloride was obtained from Eastman Kodak Co. Hemicholinium-3 (HC-3) was obtained from Aldrich Co. Carbachol (carbamylcholine) and

pilocarpine were purchased from British Drug House. Phenoxybenzamine
hodroxide (dibenzyline) and tranylcypromine were obtained from Smith
Kline and French.

2.2 Methods:

2.21 Rearing of P. americana:

The roaches were reared in glass battery jars in the culture
room at 30°C and 50 to 60% R.H. The insects were supplied with water
and rabbit pellets as food. The roaches were kept in the laboratory
first for at least 12 hours before the experiment. Only male, adult
roaches were used for the experiment.

2.22 In situ nerve cord preparation:

A male cockroach was lightly anaesthetized with carbon dioxide.
After the wings, antennae, and legs were cut off, the roach was pinned
on a wax block, slit dorsally and eviscerated. The nerve cord was
moistened with insect saline: NaCl 9.0 gm., KCl 0.2 gm., $CaCl_2$ 0.2 gm.
per liter of distilled water; pH 7.0 (Pringle, 1938). A fresh wax
block was used for each roach. Mounted on a micro-manipulator, a pair
of fine-tapered platinum electrodes was hooked under the nerve cord
between the 5th and 6th abdominal ganglia. A period of fifteen minutes
was allowed for the nerve cord to achieve its steady state (Weiant, 1958).
Stimulation of the anal cerci by air puff was accomplished by pressing
a rubber bulb connected to a 16 cm. long Pasteur pipette, the tip of
which was 0.5 cm. from, but directed toward, the left cercus. Adapted
after Colhoun (1958b, 1960), this method eliminated the possible

production of neuroactive agents by electrical stimulation reported by
Sternburg, Chang and Kearns (1959). Potentials evoked by drugs or by
preganglionic stimulation could also be detected conveniently. The
analysis of synaptic response was according to that of Prosser (1940).

Before recording the electrical activity of a nerve cord, the
bathing solution was carefully withdrawn with a Pasteur pipette, leaving
the nerve cord resting on the electrodes. Wires from the two platinum
electrodes were connected push-pull to a Tektronix Type 122 preamplifier,
from which single-ended output was connected to a Tektronix Type 502
dual-beam oscilloscope. The upper beam was used to display the electrical
activity.

A flexible copper plate was placed above, and a second one was
placed below the rubber bulb which did the air puffing. These plates
were held in place by a piece of lucite plastic. One piece of wire was
soldered to each of the copper plates; one led directly to a terminal
of a six volt radio battery and the other led to an input of the lower
beam of the oscilloscope.

From the other terminal of the battery, a wire was connected to
the grounding input of the lower beam. Whenever the bulb was pressed, a
current occurred between the two copper plates, resulting in a short
visible trace on the oscilloscope screen. This indicated the beginning of
the effect of air puffing the cerci of roaches, not the duration of the
air puff. Recording of the electrical activity was made with a Grass C-4
oscilloscope camera, using Kodak Kind-1732 photographic paper.

To reduce electrical noise from the environment, the micro-manipulator, nerve cord preparations, and the preamplifier were housed within a grounded copper-wire cage.

Diazinon, Sevin and Zectran were dissolved in Mazola corn oil (Lalonde and Brown, 1954). Eserine was first dissolved in acetone, and then diluted to the desired concentrations in insect saline with no more than one per cent acetone in the final solutions. Up to eight per cent, acetone produced no detectable effect on the AChE activity (Dauterman, Talens, and van Asperen, 1962). Each solution was applied gently by a Pasteur pipette to the 6th ganglion. After the initial observation was made, the entire nerve cord was bathed in the solution. Since the solution gradually leaked out of the roach abdomen, additional solution was applied as required.

3. Determination of AChE Activity:

The AChE was assayed by a modification of van Asperen's method (1962) which was based on Gomori's technique (1953). This sensitive method is primarily a colorimetric determination of naphthol produced by the enzymatic hydrolysis of naphthylacetate.

3.1 Materials:

Naphthol was obtained from Fisher Scientific Co., α-naphthyl-acetate from Eastman Kodak Co., and diazoblue-B from Edward Gurr, Ltd.

3.2 Methods:

3.21 Enzyme Preparation:

The 6th abdominal ganglion was dissected from an adult roach,

and rinsed in insect saline solution. The ganglion was then homogenized in ice-cold 0.01 M phosphate buffer pH 7 in a Potter and Elvehjem homogenizer. The homogenate was centrifuged at 30,000 x g and the supernatant diluted to one-half ganglion per ml.

3.22 Substrate Solutions:

Substrate solution was prepared by diluting a stock solution of α-naphthylacetate (0.03 M) in acetone with 0.01 M phosphate buffer pH 7 to give a final substrate concentration of 3×10^{-4} M.

3.23 Diazoblue-B, Sodium Laurylsulfate Solution:

This solution, used for the quantitative determination of the amount of naphthol produced, consists of 3 parts of a 1% diazoblue-B solution and 7.5 parts of a 5% sodium laurylsulfate solution. The α-naphthol reacts with diazoblue-B to give a strong blue color. Sodium-laurylsulfate immediately stops all esterase activity, and solubilizes the naphthol-diazoblue complex (van Asperen, 1962).

3.24 Assay of AChE:

Cockroach nervous system contains AChE, ali-esterase (AliE), and aryl-esterase (ArE) (Chadwick, 1963). Eserine is believed to inhibit the AChE activity completely, but not the other two esterases (Chadwick, 1963).

The assay was started by pipetting 0.5 ml. of enzyme solution (1/4 ganglion) into 5 ml. of the substrate solution. The reaction was stopped by the addition of 1 ml. of diazoblue laurylsulfate solution (DBLS). In each assay, enzymatic hydrolysis was permitted in 1 tube for 0 min. and a second tube for 10 min. at 40°C. The resulting color was read in a Beckman DU-2 spectrophotometer at 600 mμ 5 min. after addition

of DBLS. The total esterase activity (EΔO.D.) was equal to O.D. of material incubated 10 min. - O.D. of material incubated 0 min. To determine the nonspecific esterase activity, the same procedure was followed, except that the substrate solution contained eserine at a particular concentration. This gave a ΔO.D. after AChE inhibition (IΔO.D.). The AChE activity (NΔO.D.) which was calculated as NΔO.D. = EΔO.D. - IΔO.D.

The same procedure was followed to assay the AChE inhibited by TEPP when electrical activity of the roaches ceased, except that the ganglia were homogenized with a phosphate buffer (pH 7) containing 0.3% acetylcholine chloride (Colhoun, 1959a).

4.   Fluorometric Determination of Catecholamines:

Modified after that of Shore and Olin (1958), this procedure has been successful in assaying catecholamines of mammalian hearts (C. W. Nash, personal communication). The catecholamines are first oxidized to red indole derivatives which then become strongly fluorescent hydroxyindoles in the alkali.

4.1 Materials:

The distilled water used throughout this experiment was demineral-ized twice.

(1) Salt-saturated butanol: 49 g. of sodium chloride, and 2 ml. of HCl were added to 946 ml. of reagent grade n-butanol.

(2) 4% Versene (pH 6.3 - 6.5): 8 g. of EDTA were dissolved in 200 ml. of distilled water, and the pH adjusted to 6.3 - 6.5 using sodium hydroxide.

(3) 0.1 M Iodine: 0.75 g. of iodine and 14.40 g. of potassium iodide were dissolved in 300 ml. of distilled water.

(4) Alkaline sulfite solution: 0.63 g. of anhydrous sodium sulfite were dissolved in 5 ml. of distilled water, to this 20 ml. of 5 N NaOH were added.

(5) Standard noradrenaline and dopamine were obtained from New England Nuclear Corp.

4.2 Method:

The abdominal nerve cords were dissected from adult roaches, and transferred to ice-cold saline. The nerve cords were then frozen in lots of ten, and stored overnight in a deep freezer. Before the extraction procedure began, the nerve cords were thawed, taken out of the vials and frozen again by liquid nitrogen. The total weight of the nerve cords was 0.493 g. The nerve cords were homogenized in Potter and Elvehjem homogenizer with 4 ml. of cold, acid, salt-saturated butanol for 5 min. The homogenate was transferred to a 15 ml. screw-cap centrifuge tube, shaken for 5 min. and centrifuged for 10 min. at 540 x g. The supernatant was transferred to another 15 ml. screw-cap centrifuge tube, and mixed with 6 ml. of heptane and 1.5 ml. of 0.01 N HCl. The mixture was shaken and centrifuged at 540 xg for 5 min. The organic layer was discarded.

The acid layer was then pipetted in 0.5 ml. aliquots into three test tubes containing 1 ml. of 4% versene each, and 0.2 ml. of 0.1 M iodine was added to two of these tubes. After 2 minutes, 0.5 ml. of alkaline sulfite solution was added. The third tube was used as a tissue blank, which had its order of oxidation reversed, i.e., alkaline sulfite

preceding iodine. After another 2 minutes, 0.6 ml. of 5 N acetic acid was added. The contents of the tubes were heated in a boiling water bath for 5 minutes, and cooled rapidly in cold water to room temperature. The fluorescence for dopamine was read in an Aminco Bowman spectrofluorometer with activation and fluorescence wavelength at 345 mμ and 416 mμ respectively (Carlsson and Waldeck, 1958). The activation and fluorescence wavelength for noradrenaline were at 385 mμ and 485 mμ.

Two dopamine and noradrenaline standards, and two reagent blanks were run together with the sample. Each of the standards contained 0.25 μg. of the catecholamines.

III. RESULTS AND DISCUSSION

1.   The Normal Endogenous Activity:

Four roach nerve cords were treated with saline alone, and the endogenous activity was observed during an 8 hour period (Figs. 1 & 2). The synaptic transmission was generally good even at the 8th hour. The endogenous activity and synaptic transmission varied from hour to hour. The patterns of endogenous activity and synaptic transmission were almost identical although the peaks did not always coincide. The endogenous activity showed a tendency to decrease during the first hour. This might be due to the decomposition of arginine phosphate during dissection, attendant stimulation and injury of the nerve cord (Engel and Gerard, 1935), since the phosphagen of insects is arginine phosphate (Gilmour, 1965). The fluctuations in endogenous activity were also observed by Twarog and Roeder (1957). Two other nerve cords were treated with pure Mazola corn oil for eight hours (Fig. 3). The endogenous activity was lower than that of saline controls. This was possibly due to the extensive hydrolysis of arginine phosphate during anoxia (Engel and Gerard, 1935). The synaptic transmission decreased with time.

Tobias et al. (1946) demonstrated that roach nerve cords could synthesize ACh. Though excised, nerves of lobster leg were able to synthesize ACh (Dettbarn and Rosenberg, 1966).

2. The Effect of Hemicholinium (HC-3):

Hemicholinium (10^{-3} M) blocked synaptic transmission of two
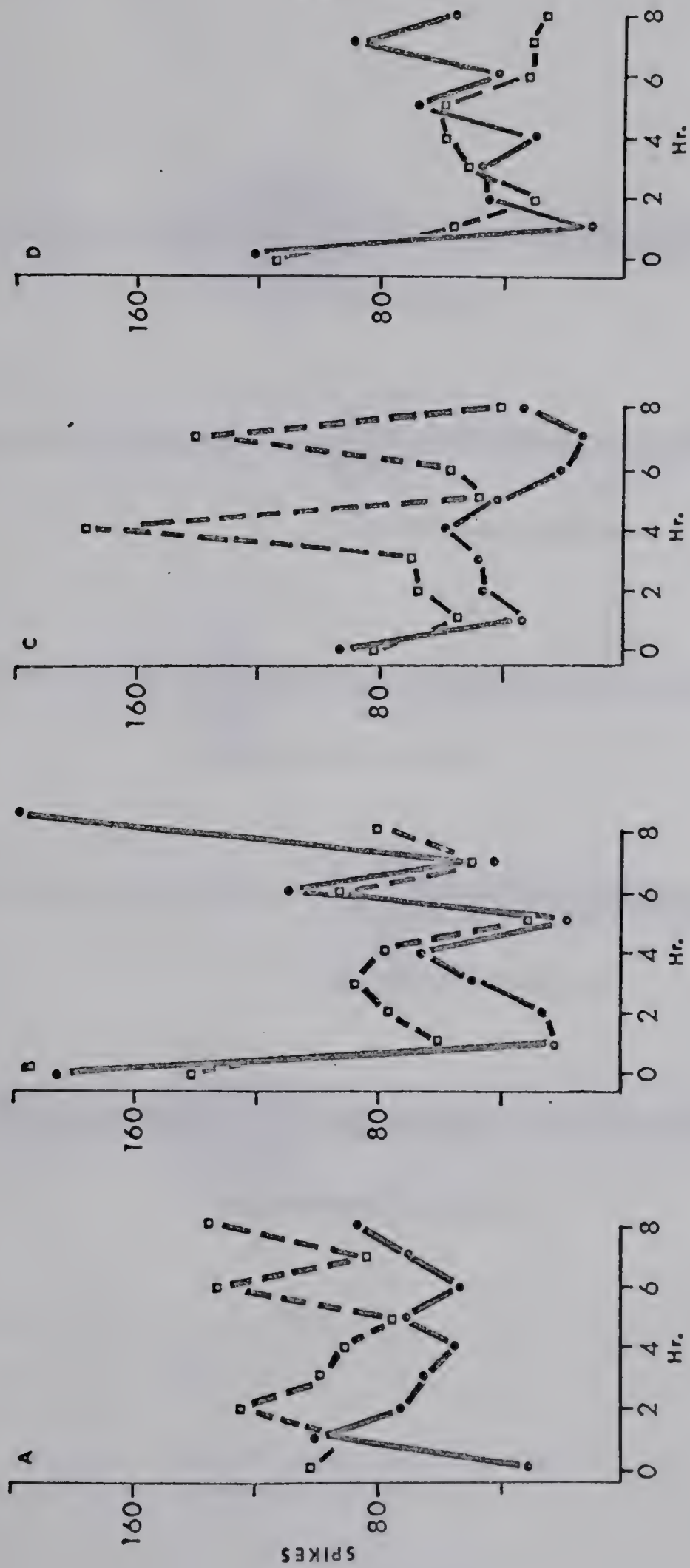
Fig. 1   Electrical activity of 4 roaches treated with insect saline

• spikes/sec.;   □ spikes/air puff
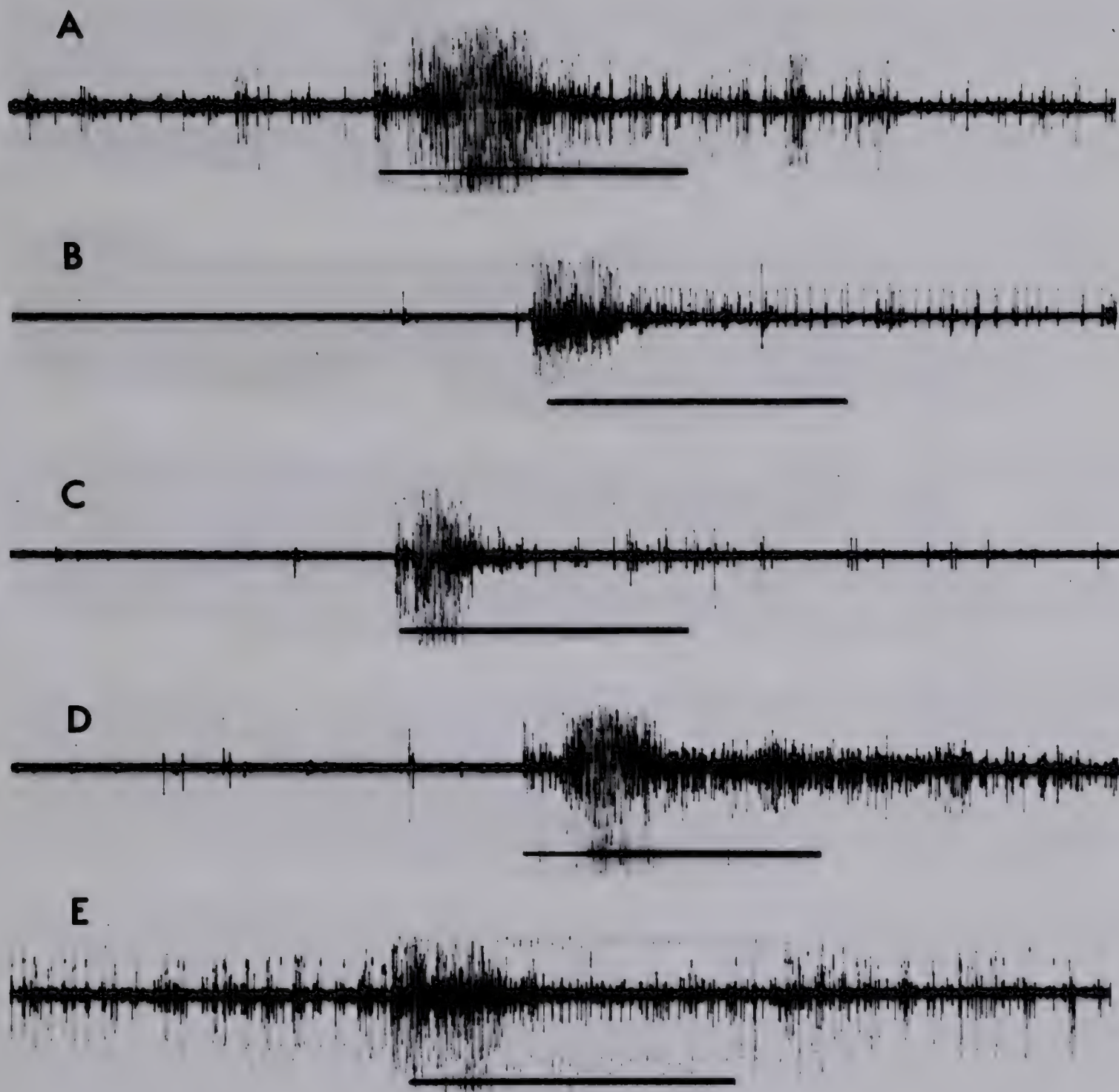
Fig. 2   Electrical activity of a saline control roach.

(A) 0 hr.;   (B) 2 hrs.;   (C) 4 hrs.;   (D) 6 hrs.;
(E) 8 hrs.

Solid line air puff response. Film speed 10 cm./sec.
Vertical scale 800 µv/1.2 cm.
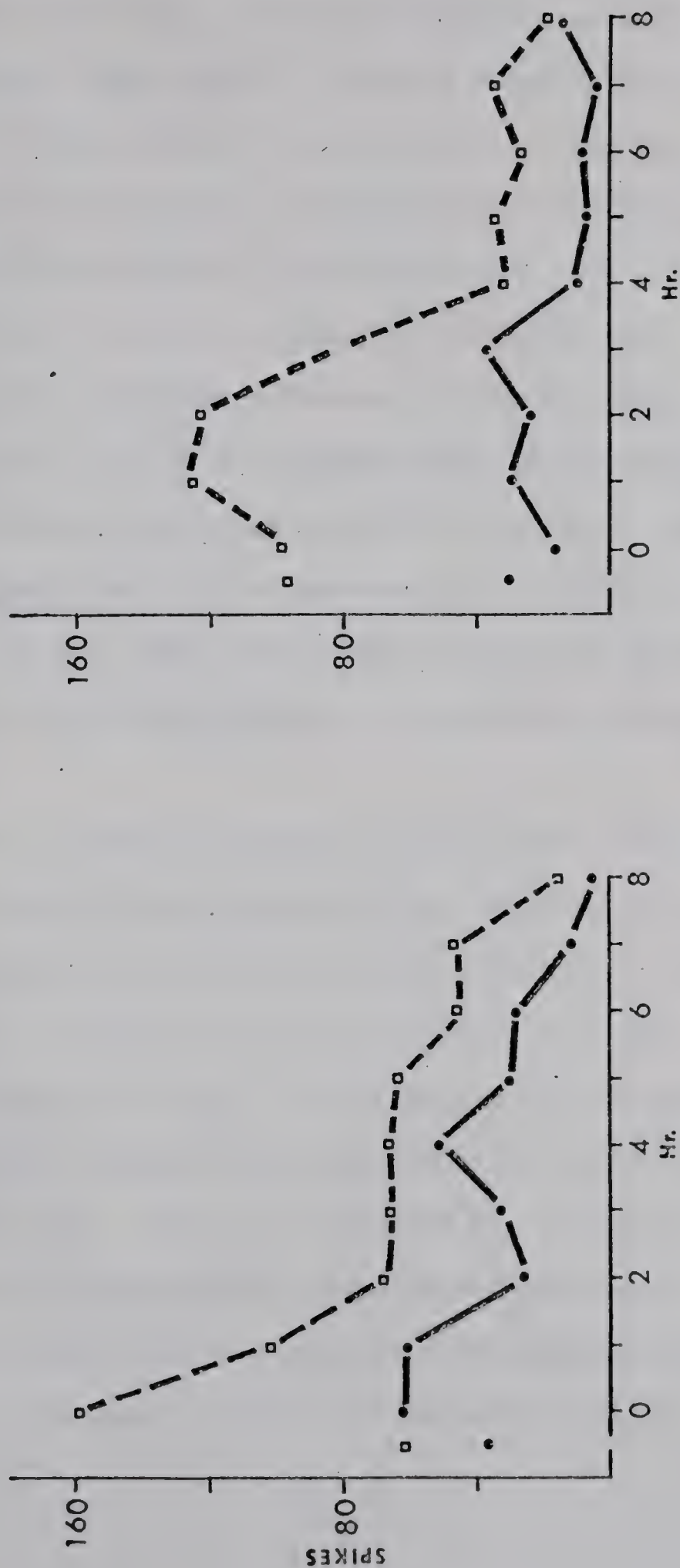
Fig. 3   Electrical activity of 2 roaches treated with Mazola corn oil.

● spikes/sec.;   □ spikes/air puff.

roaches (Fig. 4c,d), and incompletely blocked the synaptic activity of two other roaches (Fig. 4a,b). Both the endogenous activity and synaptic transmission showed steady decline. Starting after an hour or two, the amplitudes of the endogenous activity and synaptic response decreased (Fig. 5). In order to facilitate the depletion of endogenous ACh, the cerci were puffed for a period of ten minutes each hour. The endogenous activity of the four roaches was completely abolished after 1 to 5 hours.

Three other roaches were treated with hemicholinium (10^{-3} M), and choline chloride (10^{-3} M) was applied when both the endogenous activity and synaptic transmission appeared to reach the lowest level (Figs. 6, 7). Again, the activity pattern showed a decline in the presence of HC-3 alone. After the choline was applied, the endogenous activity was increased, and the synaptic transmission was greatly improved.

No previous work has been done on the effect of HC-3 on the electrical activity of insect nervous system. HC-3 is reported to interfere with the synthesis of ACh by preventing the access of choline to choline acetylase (Gardiner, 1961), or by occupation of the storage sites for ACh (MacIntosh, 1961). HC-3 inhibited the ACh synthesis in mammalian sympathetic ganglia (Birks and MacIntosh, 1961; MacIntosh, Birks and Sastry, 1956). HC-3 also inhibited the synthesis of ACh in minced mouse brain. Postganglionic sympathetic transmission was blocked by HC-3 (Burn and Rand, 1960). The block of ACh synthesis by HC-3 could be reversed by large doses of choline (Birks and MacIntosh, 1961; Gardiner, 1961).

Fig. 4   Electrical activity of 4 roaches treated with 10-3 M HC-3.

• spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.
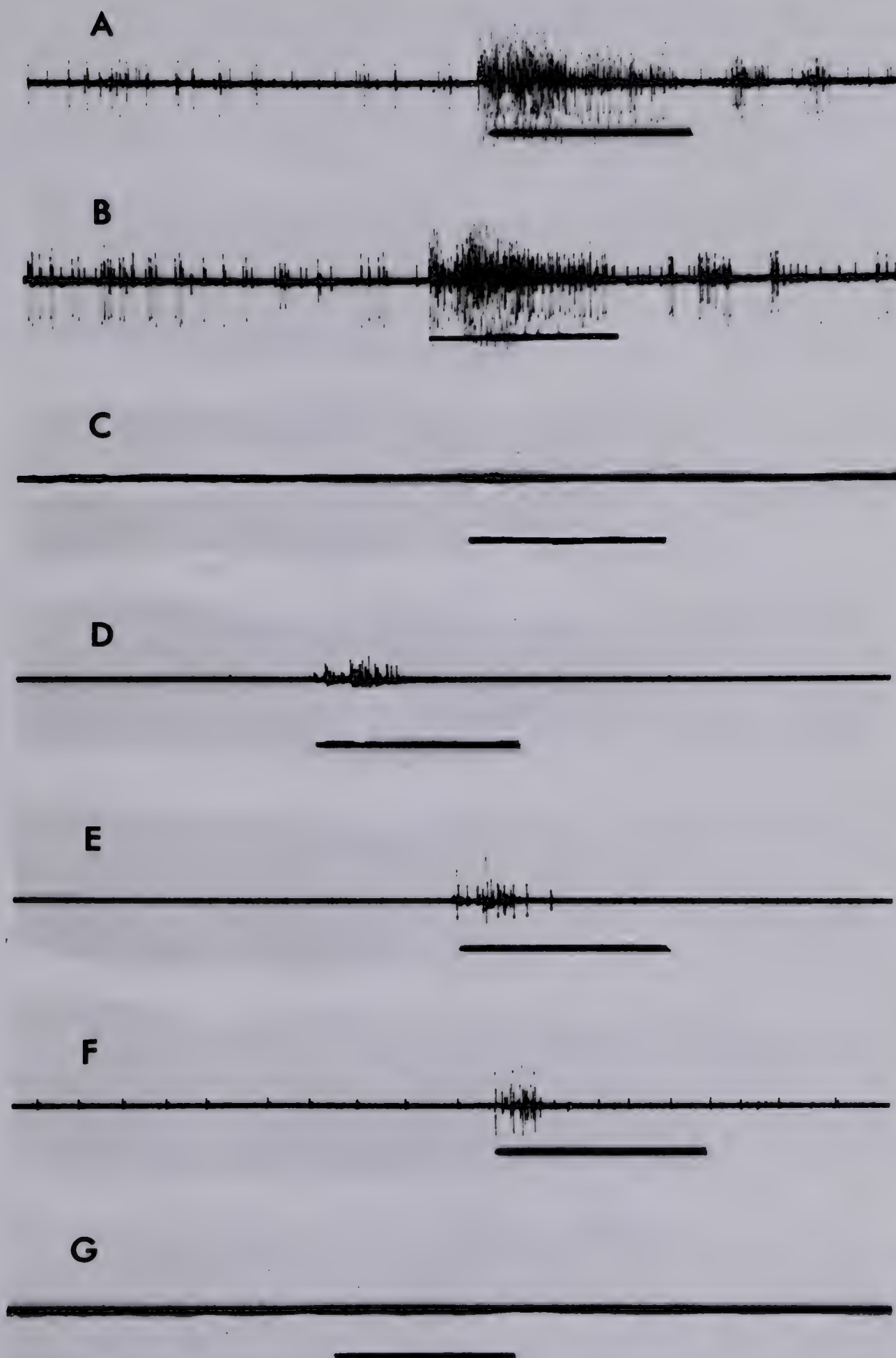
Fig.   Electrical activity of a roach treated with 10^{-3} M HC-3.

(A) before treatment;   (B) 0 hr.;   (C) 1 hr.;
(D) 2 hrs.;   (E) 3 hrs.;   (F) 4 hrs.;   (G) 5 hrs.

Solid line air puff response. Film speed 10 cm./sec.
Vertical scale 800 μv/1.2 cm.
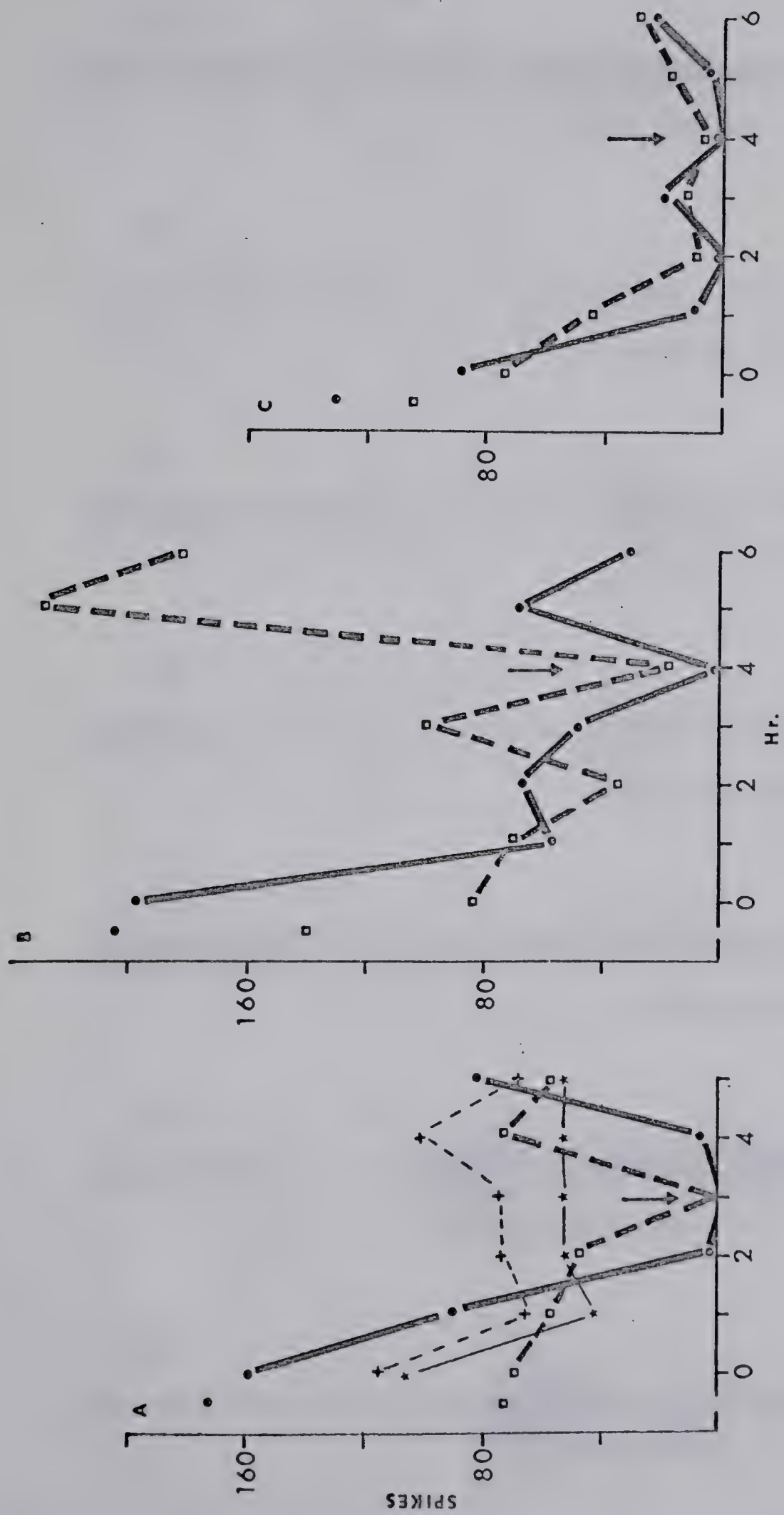
Fig. 6   Reactivation of electrical activity by 10^{-3} M choline on roaches
treated with 10-3 M HC-3.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control;
↓ choline added.
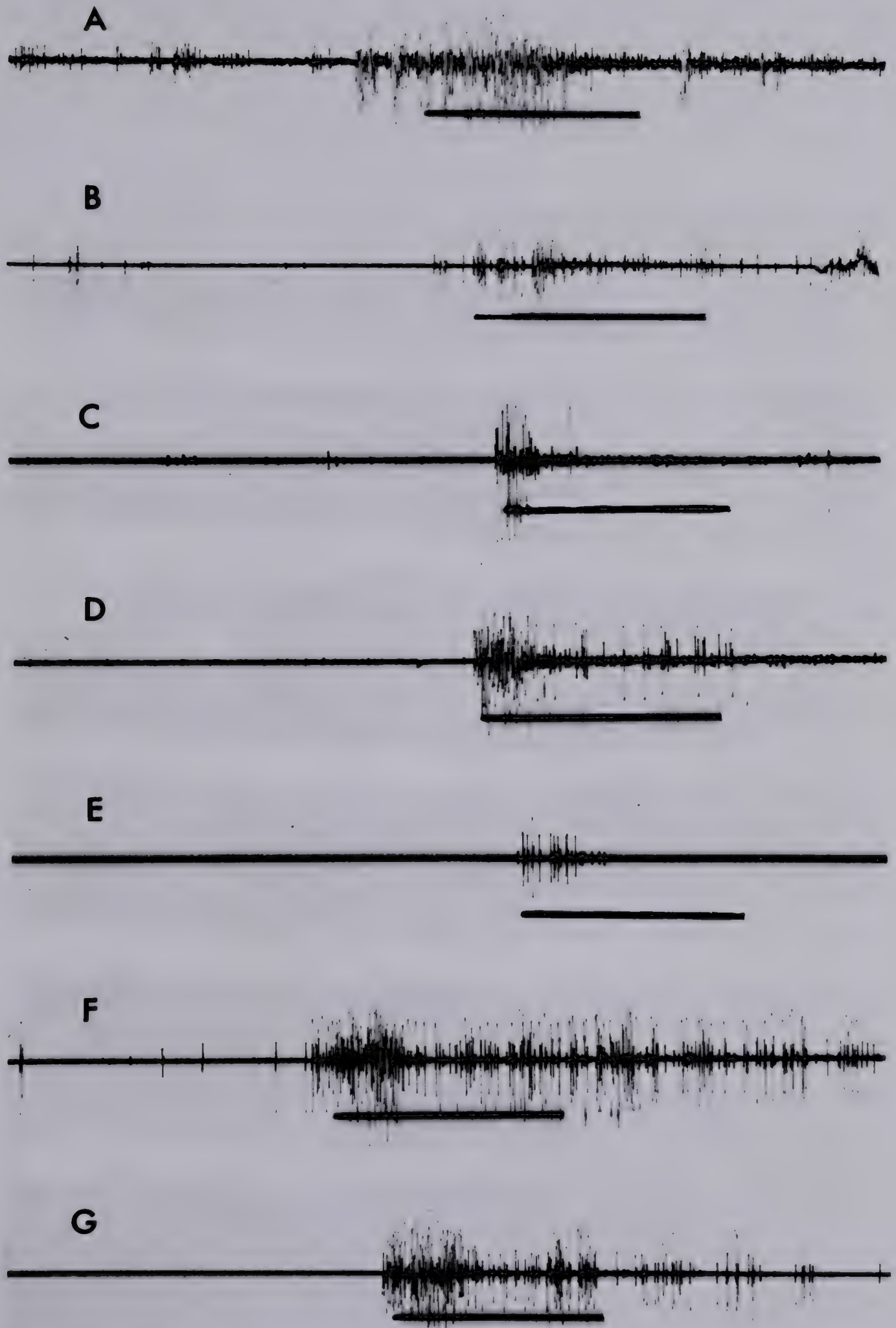
Fig. 7   Electrical activity of a roach treated with 10^{-3} M HC-3
first, and then choline.

(A) before treatment;   (B) 0 hr.; HC-3 added;
(C) 1 hr.;   (D) 2 hrs.;   (E) 3 hrs.; choline 10^{-3} added;
(F) 4 hrs.; (G) 6 hrs.

Solid line air puff response. Film speed 10 cm./sec.
Vertical scale 800 µv/1.2 cm.

The degree of inhibition of ACh synthesis by HC-3 depended upon the concentration of choline present (Gardiner, 1961). The variation in the inhibitory effect of the electrical activity in the present study could probably be explained on such basis.

3.   The Effect of Carbachol (Carbamylcholine):

Carbachol at 10^{-3} M had no observable effect on the endogenous activity and the synaptic transmission of three roaches (Fig. 8). But at 10^{-2} M, carbachol blocked both the endogenous activity and synaptic transmission in three nerve cords after approximately two hours (Fig. 9). Half an hour after the application of carbachol, the endogenous activity decreased almost to complete inactivity in one nerve cord (Fig. 9b). The spikes of synaptic transmission were barely visible. In the other two nerve cords, there was some indication that some nerve cells were acted upon. The spikes of these cells were of low amplitudes.

The results were contrary to Roeder's finding (1948) that carba-chol at 10^{-2} M had no effect on synaptic transmission in the roach's 6th abdominal ganglion. The difference might only be due to the difference in the duration of observation period involved.

Ginsborg and Guerrero (1964) reported that carbachol depolarized the sympathetic ganglion cells of the frog, and the amplitude of the spon-taneous synaptic potential was depressed five minutes after addition of carbachol. It was concluded that the depolarization was apparently a direct result of the drug acting on the receptor, and not due to a "drug induced" release of transmitter from the presynaptic fibers (Ginsborg and Guerrero,

Fig. 8   Electrical activity of 3 roaches treated with 10^{-3} M carbachol.

 • spikes/sec.; □ spikes/air puff;

 ★ average spikes/sec., saline control;
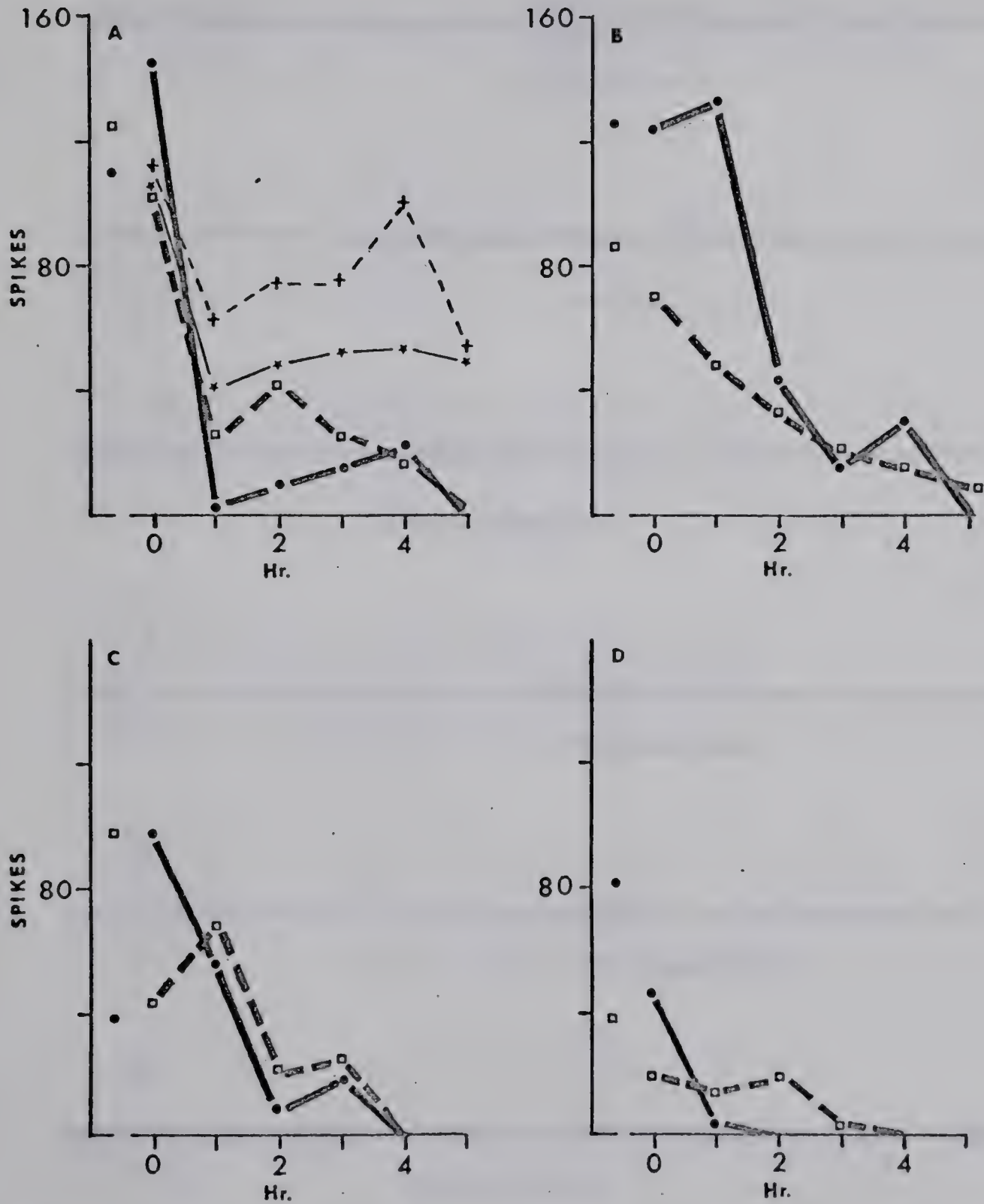
 + average spikes/air puff, saline control.

Fig. 9   Electrical activity of 3 roaches treated with 10^{-2} M carbachol.

● spikes/sec.;   □ spikes/air puff;

★ average spikes/sec., saline control;

+ average spikes/air puff, saline control.

1964). Carbachol itself is hydrolyzed much more slowly than ACh, and is completely unaffected by AChE (Barlow, 1964; Koelle, 1965).

4. The Effect of Cholinergic "Receptor-Acting" Drugs:

Ganglionic blockade generally falls into three categories:

(1) Depolarization blockade: Persisting depolarization of nerve cells causes an imbalance of sodium conductance; hence, the nerve cells cannot be depolarized any more (Woodbury, 1965; Takeshige and Volle, 1964).

(2) Hyperpolarization: Some drugs that bind onto a receptor increase the charge across the cell membrane, rendering the nerve cells harder to be depolarized (Ruch and Patton, 1965); Takeshige and Volle, 1964).

(3) Receptor blockade: Compounds that compete for the same receptor site with an endogenous transmitter but are unable to produce depolarization are called receptor inhibitors (Bartels, 1965).

4.1 The Effect of Nicotine:

Since nicotine can mimic ACh in cholinergic synapses and junctions (Albert, 1965), it was used so that the stimulatory and blocking effect of other drugs of the same category could be compared. As reported by others (Roeder and Roeder, 1939; Welsh and Gordon, 1947), nicotine (10^{-3} M) caused an immediate stimulation upon application, and then blocked synaptic and endogenous activity irreversibly. Since the effect was immediate, a graph is not presented.

4.2 The Effect of Dimethylphenylpiperazinium (DMPP):

DMPP produced no observable effect even at 10^{-2} M (Figs. 10, 11).

Fig. 10   Electrical activity of 4 roaches treated with 10^{-2} DMPP.

• spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.

Fig. 11   Electrical activity of DMPP-treated roaches.

A & B: 10^{-2} M DMPP;   C & D: 10^{-3} M DMPP.

• spikes/sec.;   ◻ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.

The electrical activity pattern was similar to that of the saline controls.

No previous work has been done on the effect of DMPP on the electrical activity of insects. DMPP is a selective stimulant of autonomic ganglion cells in vertebrates, although its blocking effect is less potent than nicotine (Chen and Portman, 1954; Chen, Portman, and Wickel, 1951; Leach, 1957).

4.3 The Effect of Methacholine (Acetyl-β-methylcholine):

Methacholine at 10^{-2} M did not produce any observable effect on the electrical activity of the roach nerve cords within a period of five hours (Fig. 12). Methacholine is equiactive as ACh in vertebrates at the postganglionic parasympathetic nerves (Albert, 1965; Bebbington and Brimblecombe, 1965). Geber and Volle (1965) observed that methacholine was even more potent than ACh as a depolarizing agent in the sympathetic (superior cervical) ganglion of the cat. Methacholine may also produce ganglionic hyperpolarization by a direct action on the sympathetic ganglion cells (Volle, 1965).

Since methacholine is less readily hydrolyzed than ACh (Grollman, 1960), the failure of methacholine to affect synaptic transmission is probably not due to its destruction by hydrolysis. Roeder (1948b) also showed that methacholine was ineffective in stimulating or blocking synaptic transmission in the roach.

4.4 The Effect of Pilocarpine:

Pilocarpine at 10^{-3} M showed a definite depressing effect on both the endogenous activity and synaptic transmission (Fig. 13). The

Fig. 12   Electrical activity of 4 roaches treated with 10^{-2} M methacholine.

● spikes/sec.;   □ spikes/air puff/
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.
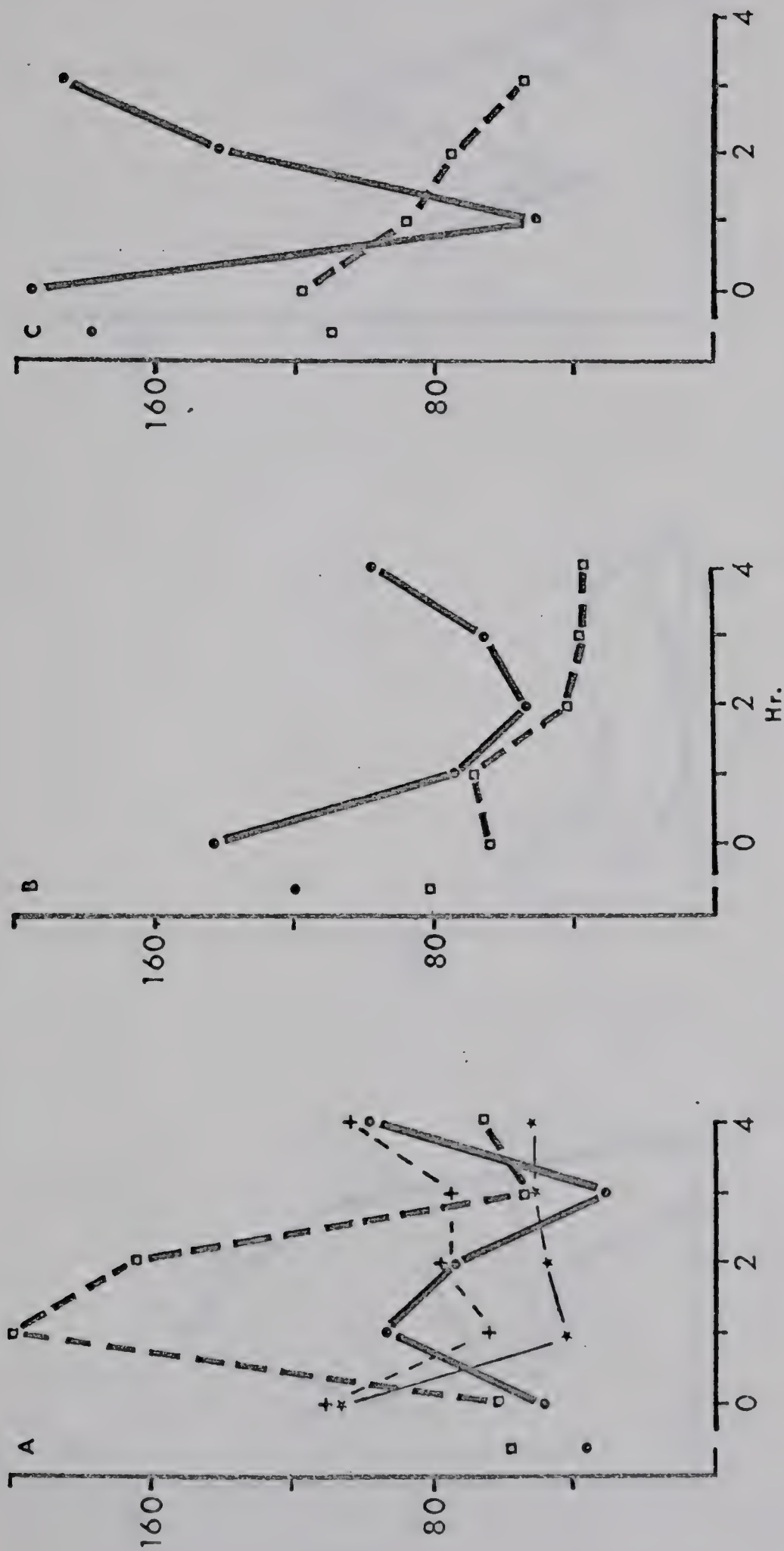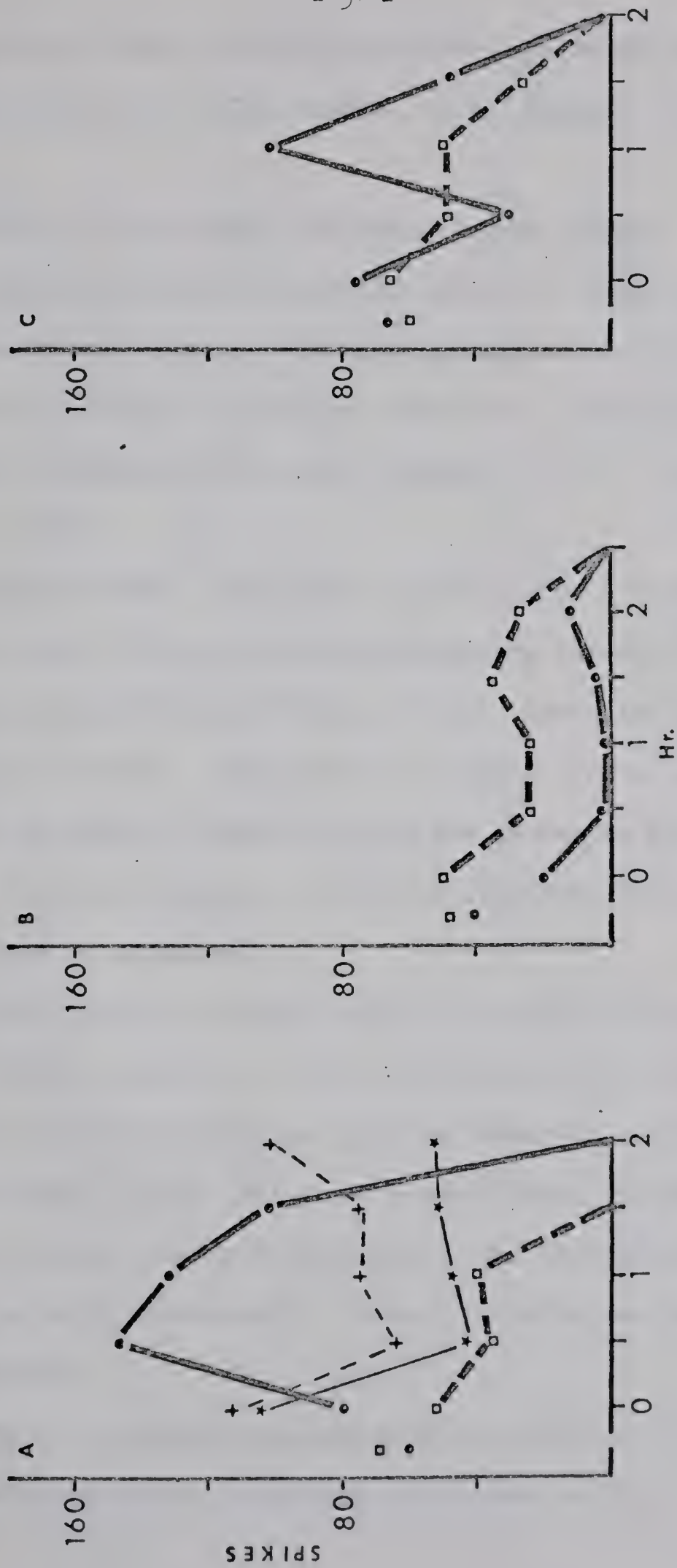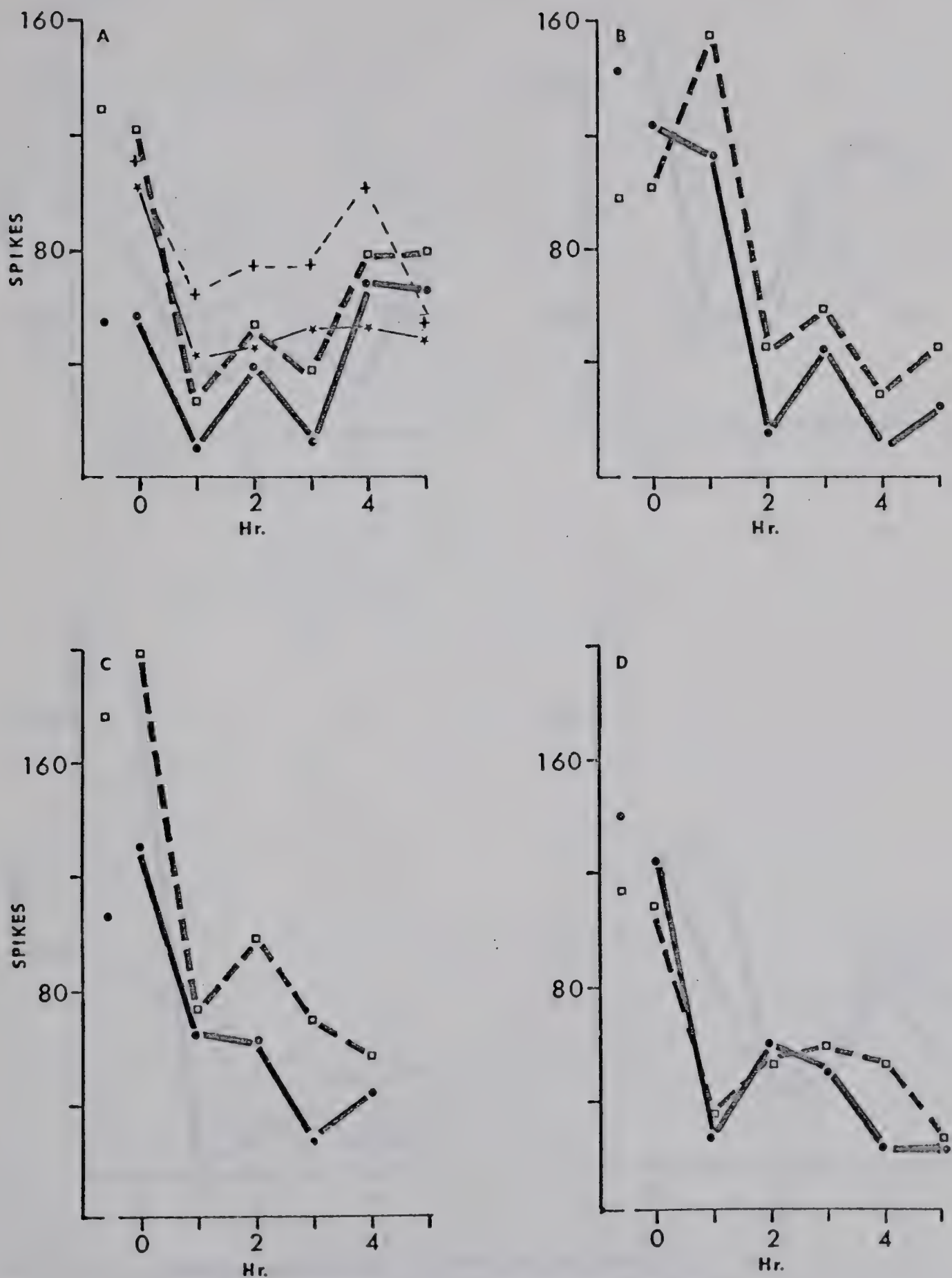
Fig. 13   Electrical activity of 4 roaches treated with 10^{-3} M
pilocarpine.

- spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
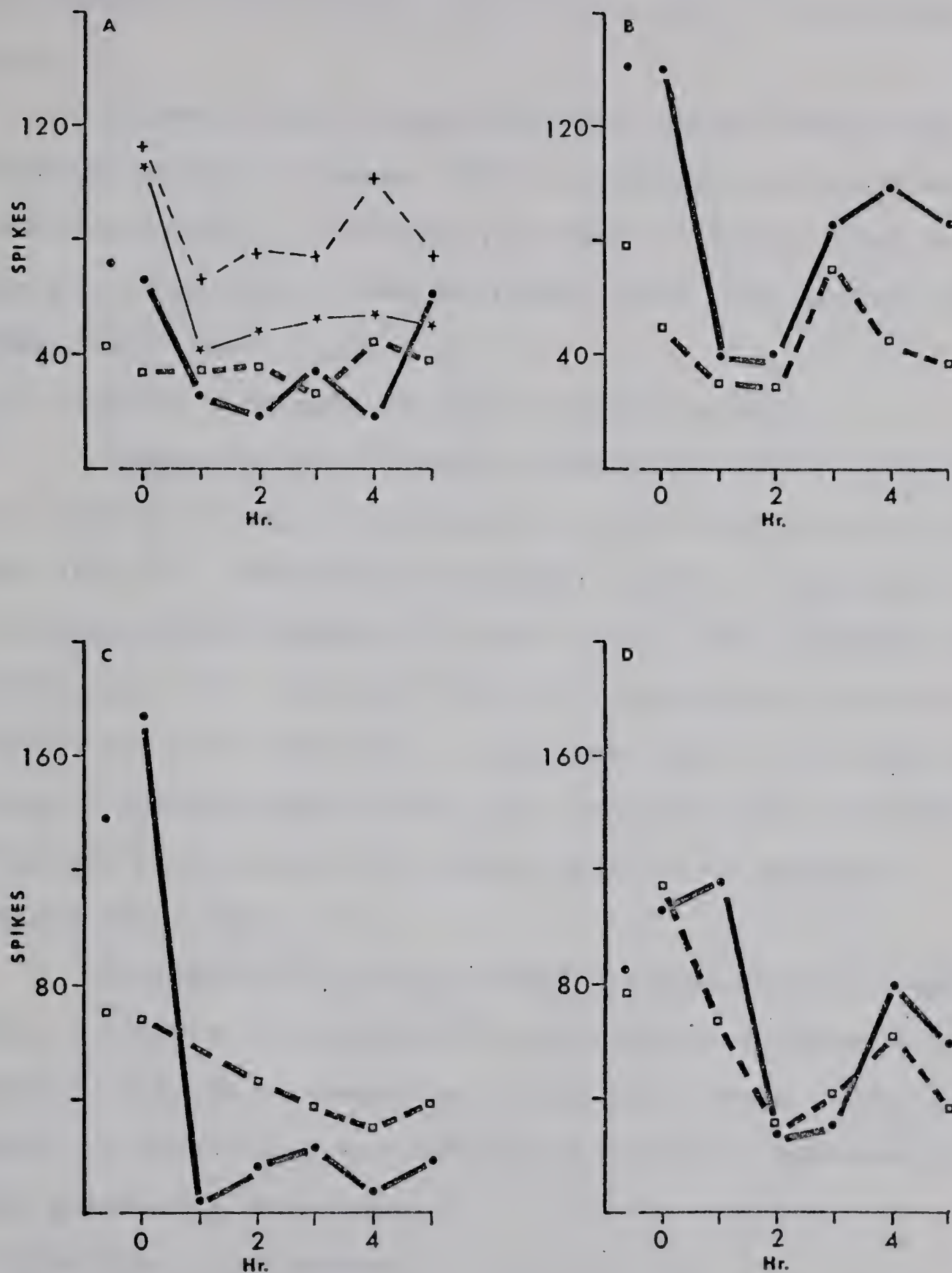+ average spikes/air puff, saline control.

amplitudes of the spikes decreased with time. The endogenous activity

and synaptic transmission were both blocked in one preparation (Fig.

13d). Some low amplitude spikes appeared after one hour, indicating

some cells were acted upon. These low amplitude spikes resembled those

induced by carbachol. Twarog and Roeder (1957) also observed such effects

from nerve cords, the ganglia of which were desheathed.

Although pilocarpine is chiefly a powerful parasympathomimetic

agent, stimulating the organs innervated by postganglionic fibers, it can

also exert ganglionic stimulation (Koelle, 1965; Grollman, 1960).

Pilocarpine is a weak competitive inhibitor of fly-head AChE

in vitro, and does not exert any progressive inhibition (Chadwick, 1964).

The results of the present investigation support the conclusion of

Chadwick (1964) that inhibition of AChE is not involved in the poisoning

of roaches by pilocarpine.

Pilocarpine injected into the heads of praying mantis produced a

state of great excitation. But when injected into the roaches, it pro-

duced immobility and apparent paralysis (Roeder, 1939).

4.5 The Effect of Tremorine:

Tremorine (10^{-2} M) depressed the endogenous activity and synaptic

transmission. The synaptic transmission was partially blocked in two out

of three preparations (Fig. 14). Beginning at the first hour, all three

preparations showed partial blocking effect with low amplitude spikes.

Some bursts of antidromic, low voltage spikes appeared at the second or

third hour. The electrical activity of one preparation was entirely

blocked.

Fig. 14 Electrical activity of 3 roaches treated with $\cdot 10^{-2}$ M Tremorine.

● spikes/sec.; □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control;

No previous work has been done on the effect of Tremorine on the electrical activity of insects. Equal in potency to ACh, Tremorine acts at the postganglionic cholinergic receptors of mammals (Blockus and Everett, 1957; Cho, Haslett, and Jenden, 1962; Everett et al., 1956). Tremorine is also a cholinomimetic agent in some central neurons (Koelle, 1965). Tremorine is believed to be metabolized first to oxotremorine (Cho, Haslett and Jenden, 1962) which then acts by forming ACh (G. M. Everett, personal communication).

4.6 The Effect of Acetylcholine:

ACh (10^{-3} M) produced no observable effect within a period of five hours (Fig. 16). But at 10^{-2} M ACh blocked both the endogenous activity and synaptic transmission in five out of six preparations (Figs. 15, 16). The stimulatory effect of ACh observed in mammalian ganglia (Geber and Volle, 1965); Takeshige and Volle, 1962, 1963; Volle, 1965) was not observed in the roaches. ACh exhibited a progressive depressing effect (Fig. 17). Three eserinized nerve cords (eserine 10^{-5} M) were washed with saline when synaptic after-discharge occurred, and then 10^{-4} M, 10^{-3} M, 10^{-2} M ACh was applied to them immediately. No apparent stimulation was observed. The endogenous activity and synaptic transmission approached normal within a period of thirty minutes.

Roeder and Roeder (1939) reported that 10^{-3} M ACh produced a definite increase in the level of endogenous activity in isolated nerve cords. But when the nerve cords were in situ, ACh 10^{-2} M had no effect. (Twarog and Roeder, 1957). If the ganglia were desheathed and eserinized, 10^{-3} M ACh caused a partial synaptic block, and in concentrations
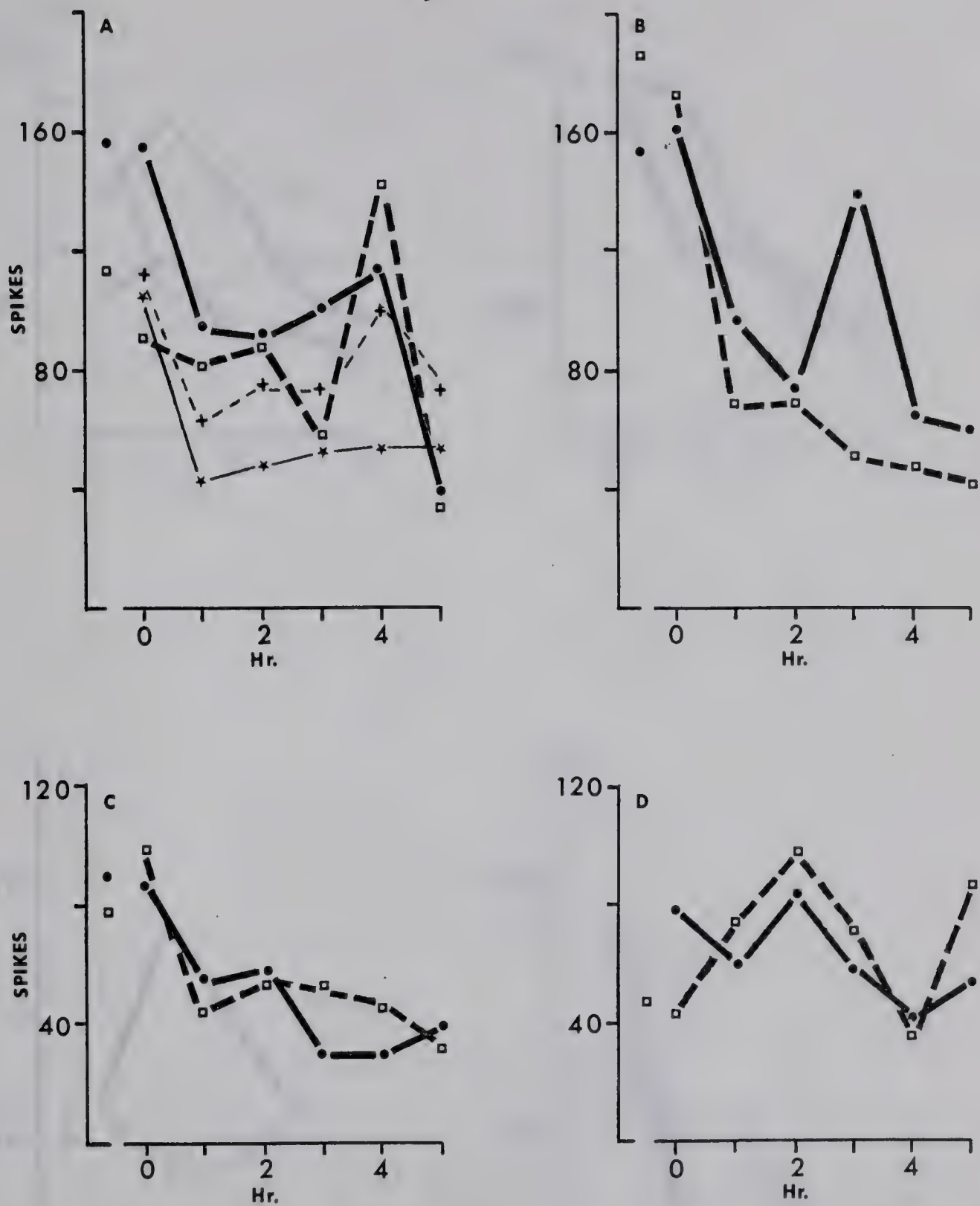
Fig. 15   Electrical activity of 4 roaches treated with 10^{-2} M ACh.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
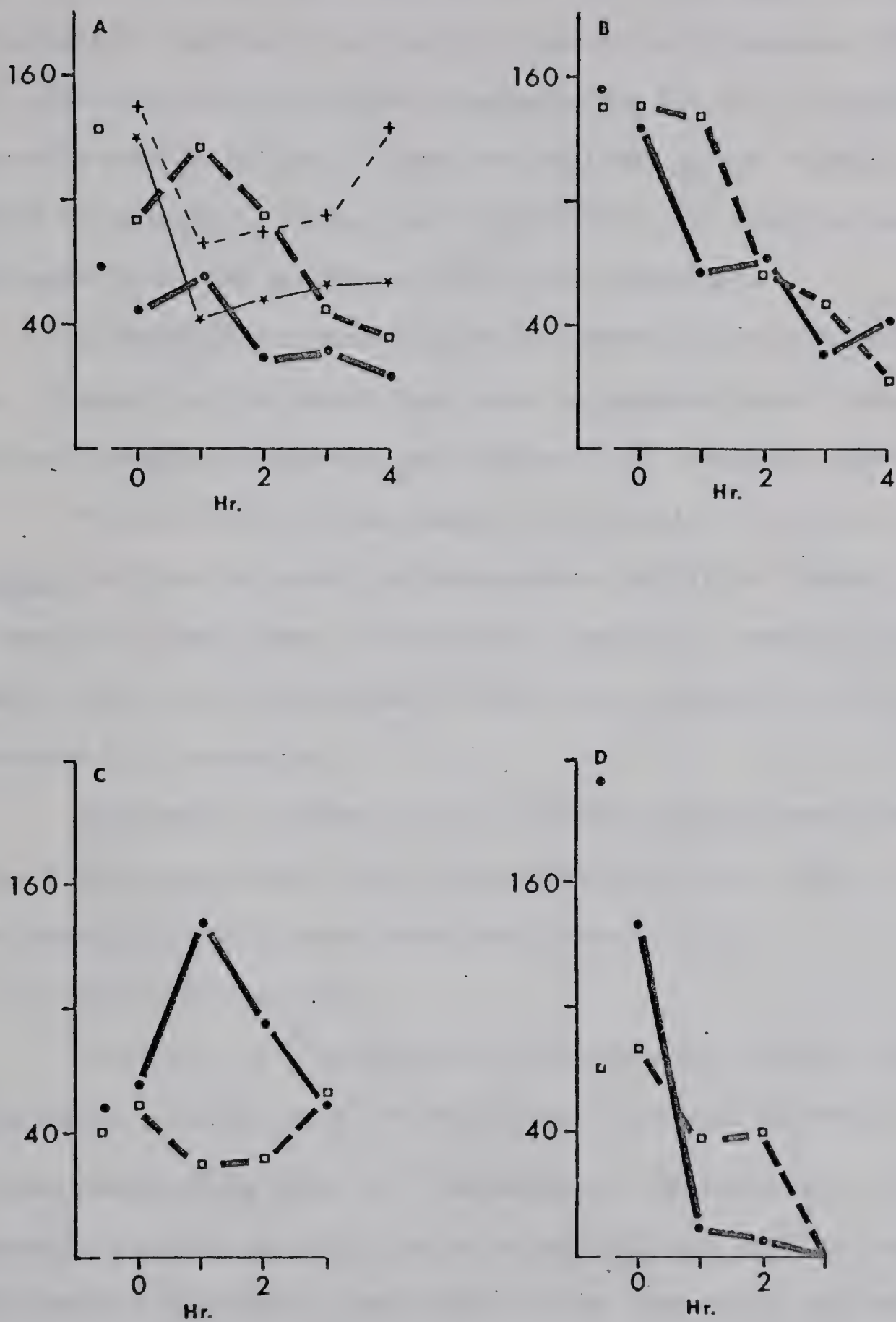+ average spikes/air puff, saline control.

Fig. 16   Electrical activity of ACh-treated roaches.

A & B: 10^{-2} M ACh;   C & D: 10^{-3} M ACh.

● spikes/sec.;   ▢ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control;
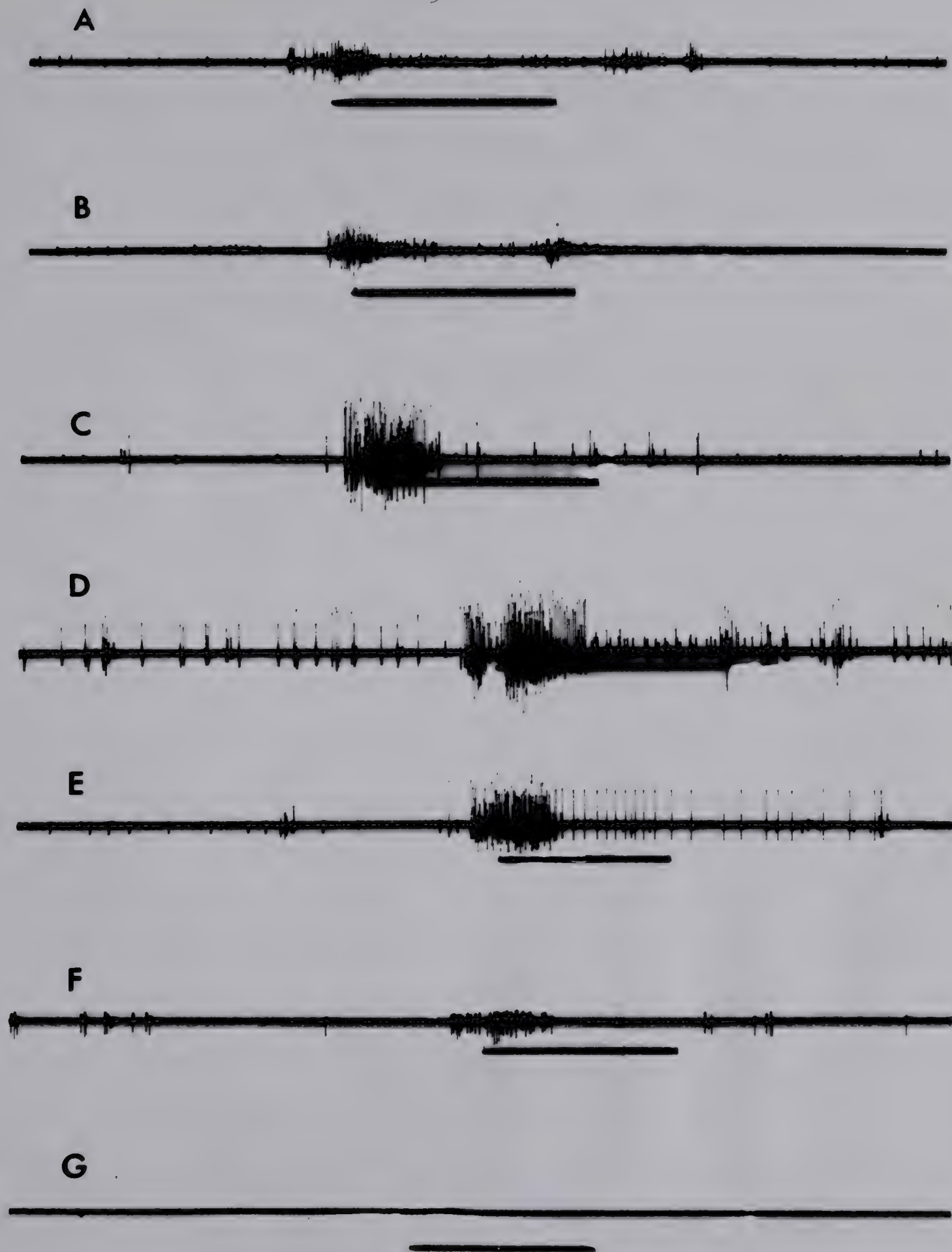
Fig. 17   Electrical activity of a roach treated with 10^{-3} M ACh.

(A) before treatment;   (B) 0 hr.;   (C) 1 hr.;
(D) 2 hrs.;   (E) 3 hrs.;   (F) 4 hrs.;   (G) 5 hrs.

Solid line air puff response.   Film speed 10 cm./sec.
Vertical scale 800 μv/1.2 cm.

between 3×10^{-3} M and 5×10^{-3} M, most preparations showed brief after-discharge followed by block; some preparations were incompletely blocked (Twarog and Roeder, 1957). Yamasaki and Narahashi (1960) claimed that 10^{-2} M ACh was effective in depolarizing isolated roach ganglia. They also found that if the ganglia were desheathed and eserinized, ACh was effective at concentrations as low as 10^{-4} M; but 10^{-5} M had little effect. However, Takeshige and Volle (1964) observed that when small doses of ACh were applied to eserinized cat's sympathetic ganglia, hyperpolarization occurred also, in addition to depolarization caused by a larger dose of ACh (Takeshige and Volle, 1962).

4.7 The Effect of Choline:

Choline (10^{-2} M) blocked synaptic transmission in four out of six preparations (Figs. 18, 19). Both the endogenous activity and synaptic transmission of one preparation was blocked. The endogenous activity of the other five preparations was unimpaired. In some preparations, regular medium-high and low amplitude spikes occurred. In doses three to four times greater than ACh, choline caused a low amplitude, but sustained, depolarization of the cat's sympathetic ganglia (Teker and Volle, 1965). Choline accelerated the rate of failure of post-ganglionic firing in cat's sympathetic ganglion (Volle and Koelle, 1961).

5.0 The Effect of Anticholinesterases:

The active center of AChE apparently has two sites, an anionic site and an esteratic site (Nachmansohn and Wilson, 1951; Wilson, 1960). Compounds which can react with one or both of these sites can inhibit or inactivate AChE, and are called anticholinesterases (Koelle, 1965).
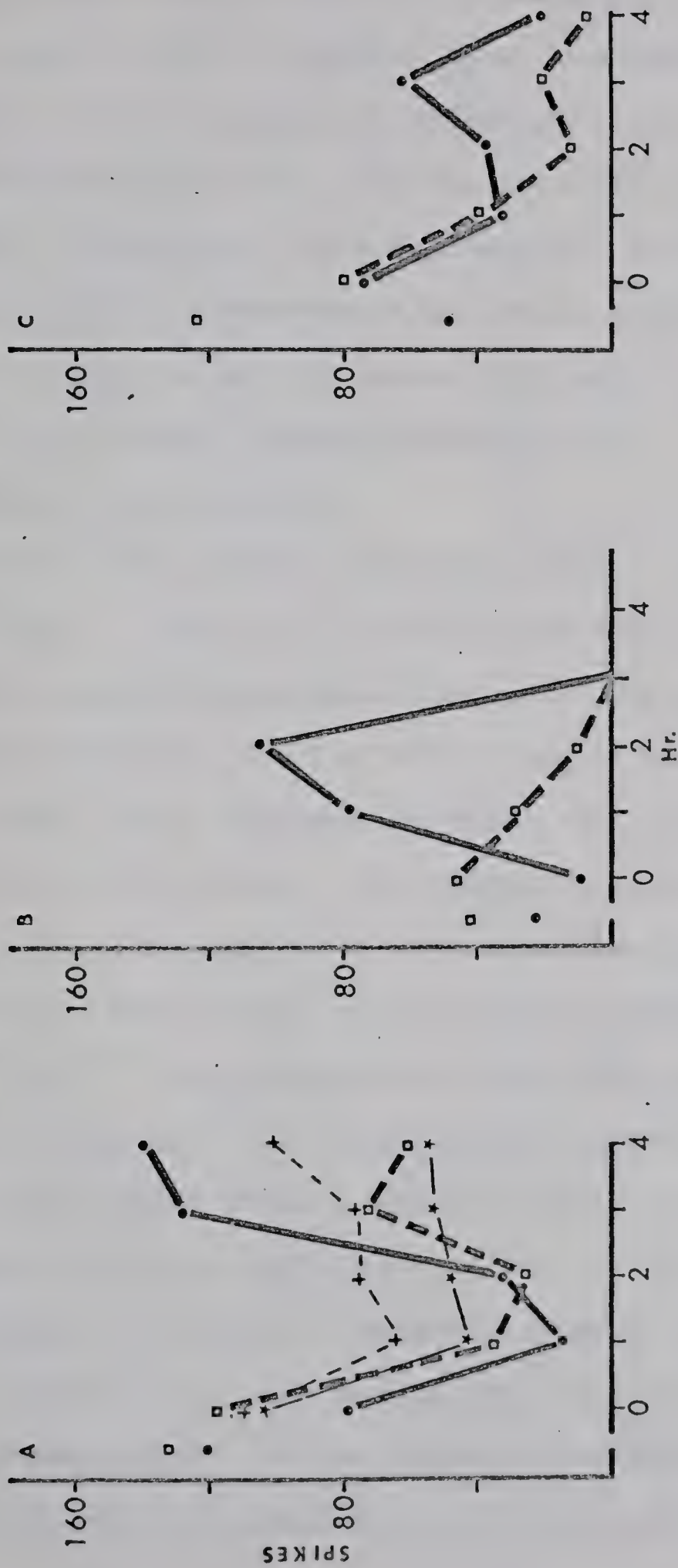
Fig. 18   Electrical activity of 3 roaches treated with 10^{-2} M choline.

• spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.
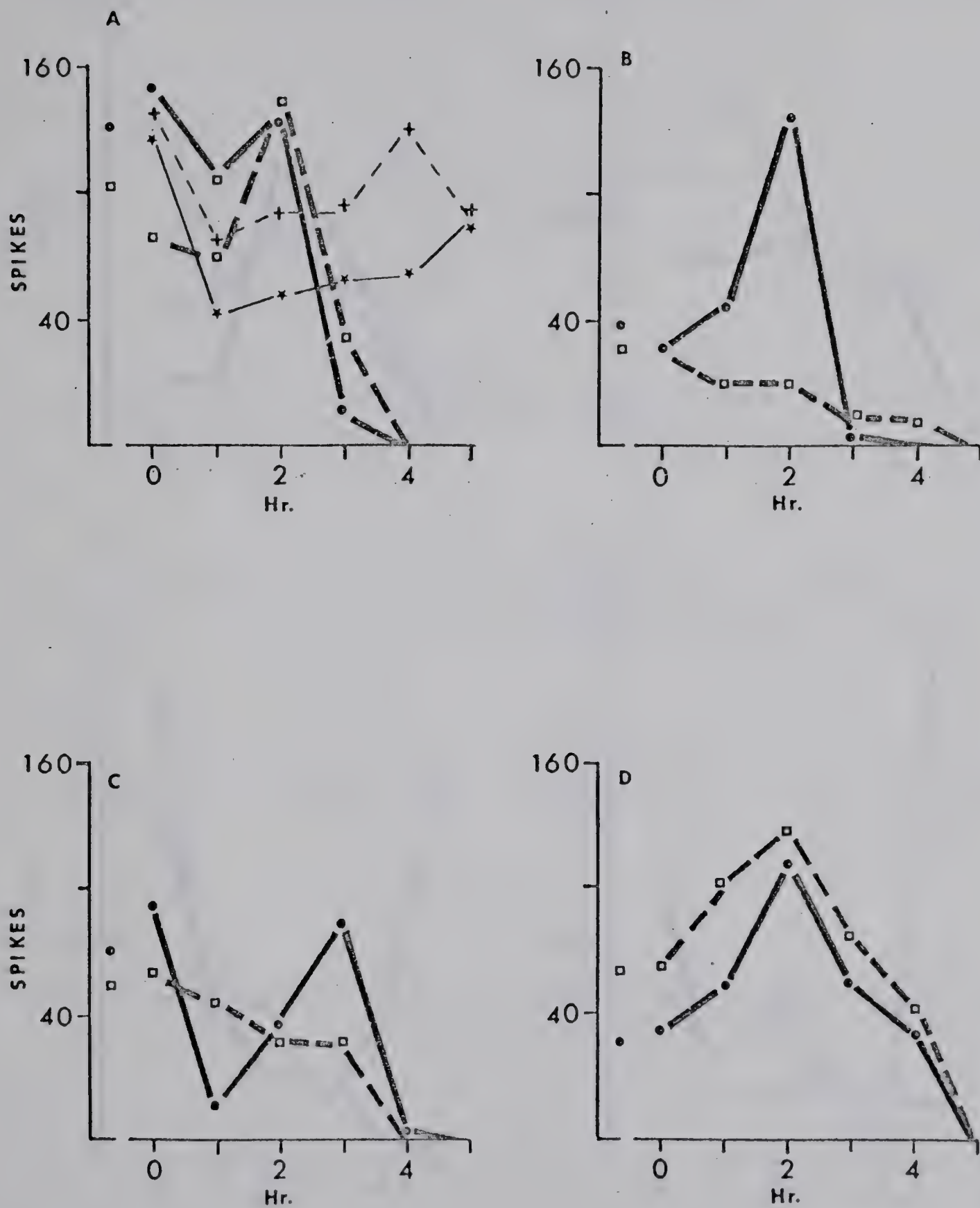
Fig. 19   Electrical activity of 3 roaches treated with 10^{-2} M choline.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
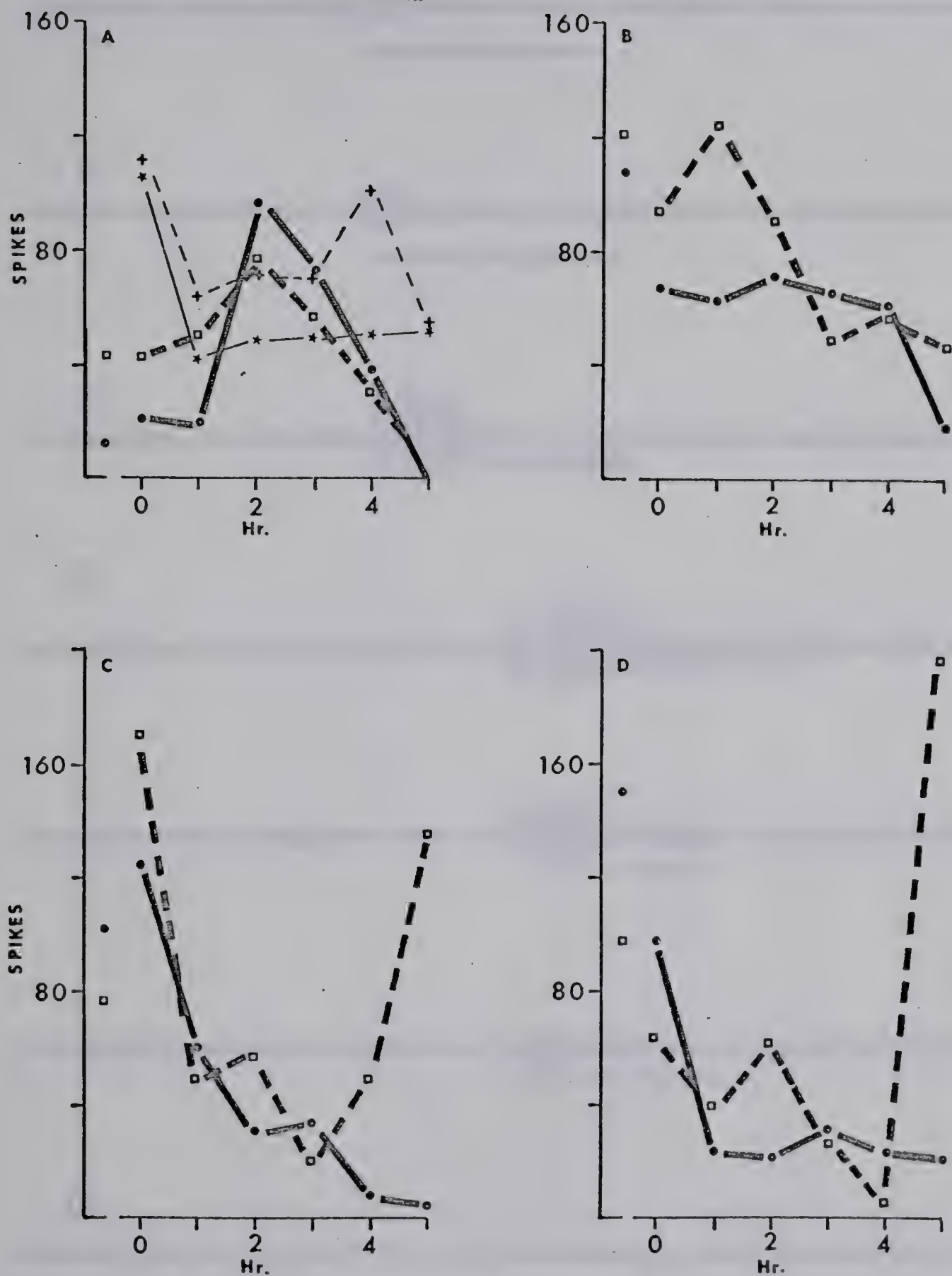+ average spikes/air puff, saline control.

Anticholinesterases cause endogenous ACh to accumulate at cholinergic sites in vivo, thus potentially capable of producing effects equivalent to continuous stimulation of cholinergic fibers (Koelle, 1965). Anticholinesterases may be either reversible or irreversible inhibitors. Carbamates are reversible inhibitors which react with the anionic site, and/or the esteratic site of AChE (Koelle, 1965). Organophosphates are irreversible inhibitors that react with the esteratic site of AChE to form a phosphorylated enzyme (Koelle, 1965).

5.1 The Effect of Carbamates:

5.11 The Effect of Eserine:

Eserine at 10^{-7} M produced no observable effect. But anticholinesterase effects were observed with 10^{-5} M eserine about one hour after treatment; these effects consisted of antidromic bursts of low amplitude spikes and synaptic after-discharge in response to a single puff, i.e., facilitation. Washing the preparation with saline abolished these effects. These observations are in agreement with the observations of Roeder (1948b), and Yamasaki and Narahashi (1960). The low amplitude spikes increased in frequency and magnitude with time. A synaptic block sometimes developed after the onset of after-discharge caused by a single puff. Since the effect of eserine is well established, a graph was not presented.

When the in situ ganglion was desheathed, Twarog and Roeder (1957) found that eserine at 10^{-5} M was effective within five minutes, compared with ten to forty minutes in intact but isolated nerve cords (Yamasaki and Narahashi, 1960).

5.12 The Effect of Sevin:

About five minutes after the Sevin (10^{-3} M) was applied to the nerve cords, a train of high frequency and high magnitude spikes occurred, lasting as long as 10 to 35 seconds. Following such antidromic discharge, a period of complete electrical quiescence was observed, during which air puffs were unable to elicit a synaptic response. The time for recovery from the synaptic block varied. Prolonged repetitive discharge alternated with synaptic block. Complete synaptic block occurred after three to four hours (Fig. 20). Sevin at 10^{-4} M did not produce stable synaptic block within five hours.

5.13 The Effect of Zectran:

Zectran at 10^{-3} M exhibited properties essentially the same as Sevin (Fig. 21).

5.2 The Effect of Organophosphates:

5.21 The Effect of Tetraethylpyrophosphate (TEPP):

TEPP (10^{-4}M) exerted a rapid and pronounced effect on the nerve cords. Within a minute of application, repetitive discharge occurred. The duration of the discharge varied from 22 to 60 seconds. A synaptic block and an electrical quiescent period followed the initial discharge. Four out of six nerve cords did not show any recovery from the completely inactive state when washed with saline. The "endogenous" activity of one preparation reappeared for four hours after an initial quiescent period, but air puffs could not elicit a synaptic response (Fig. 22a). However, in one preparation, both the "endogenous" activity and the synaptic transmission persisted, despite the alternation of electrical quiescence

Fig. 20 Electrical activity of 3 roaches treated with 10^{-3} M Sevin.

• spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., oil control;
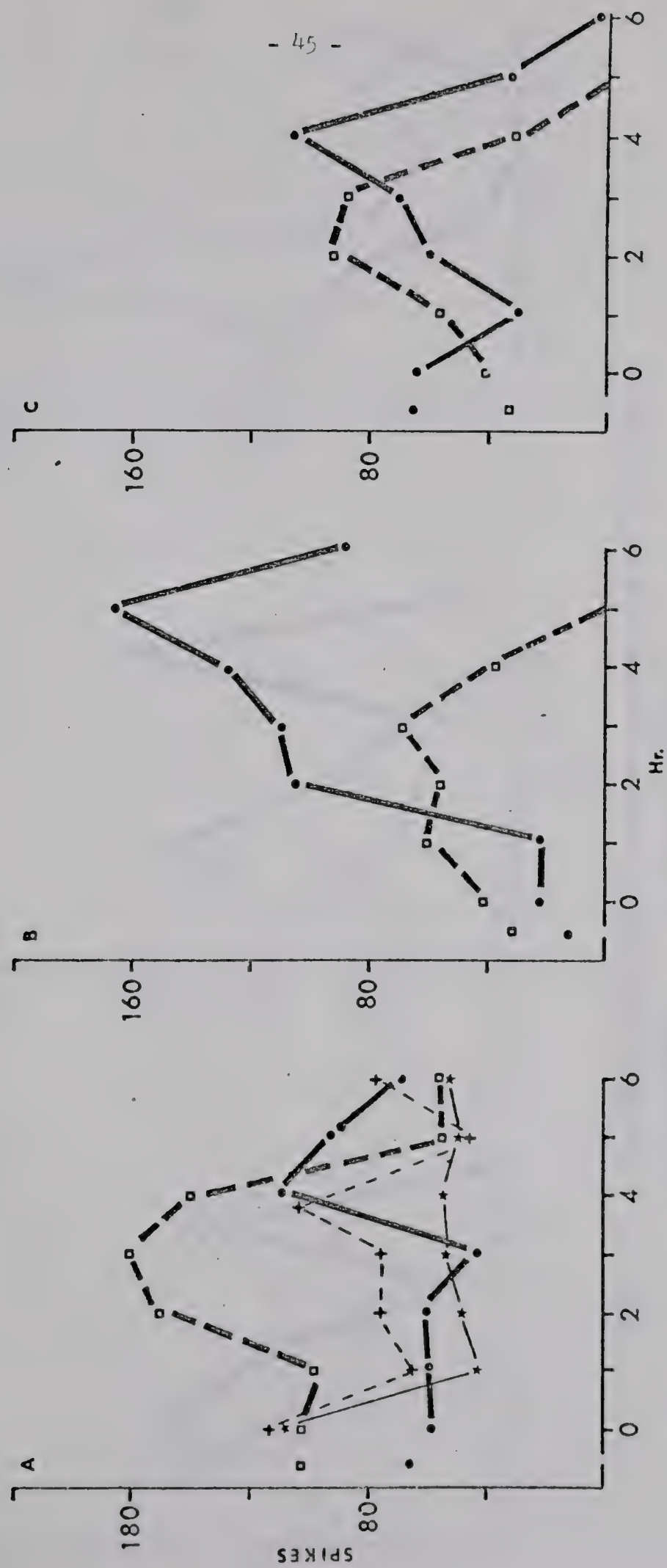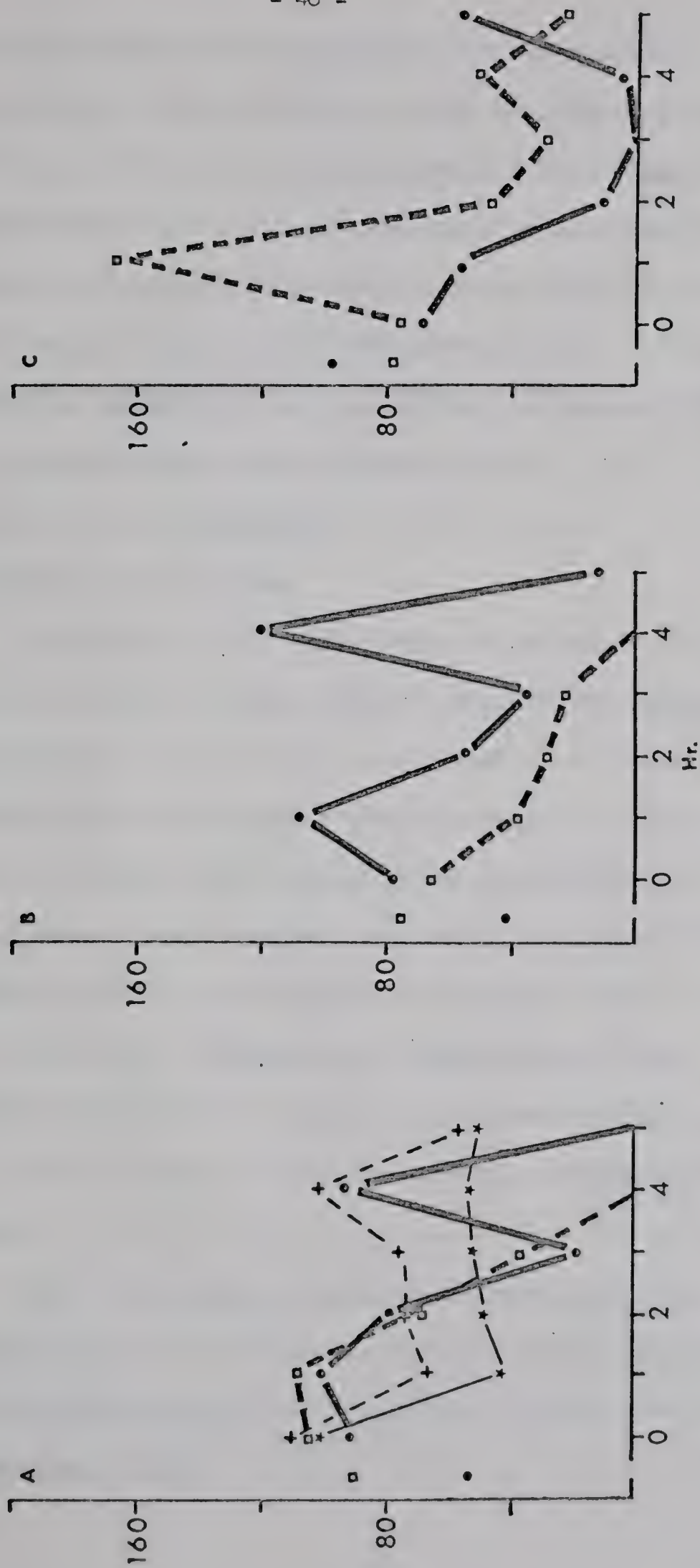+ average spikes/air puff, oil control.

Fig. 21   Electrical activity of 3 roaches treated with 10^{-3} M Zectran.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.

and synaptic block (Fig. 22b).

TEPP was reported to block synaptic transmission in nerve cords that were in situ (Colhoun, 1960; Roeder, 1948b), and in nerve cords with desheathed ganglia (Yamasaki and Narahashi, 1960). Sternburg et al. (1959) proposed that TEPP caused a neuroactive substance to be released as a result of synaptic facilitation, and this rather than TEPP was responsible for blocking the electrical activity. They also suggested that the same substance participates in neurotransmission within the central nervous system of roaches. The number of replicable observations was not indicated.

5.22 The Effect of Pyridine-2-aldoxime Methiodide (2-PAM) upon TEPP-
Treated Nerve Cords:

When complete electrical blockade was observed in four nerve cords, two treated with 10^{-4} M TEPP, two with 10^{-3} M TEPP, 2-PAM (10^{-3} M) was applied to them (Figs. 23, 24). Both the endogenous activity and synaptic transmission reappeared at the first hour, with the occurrence of facilitation and quiescence. With the exception of one preparation, synaptic after-discharge and electrical quiescence stopped at the third hour. The electrical activity of all four appeared normal at the fourth hour.

When applied alone, 10^{-3} M 2-PAM had no apparent effect on the electrical activity of the nerve cords (Fig. 25). In mammals, 2-PAM is a potent neuromuscular blocking agent (Holmes and Robins, 1955).

Phosphorylated AChE can be reactivated by 2-PAM effectively (Hobbiger, 1963). Loomis (1956), and Wilson and Ginsburg (1958) suggested
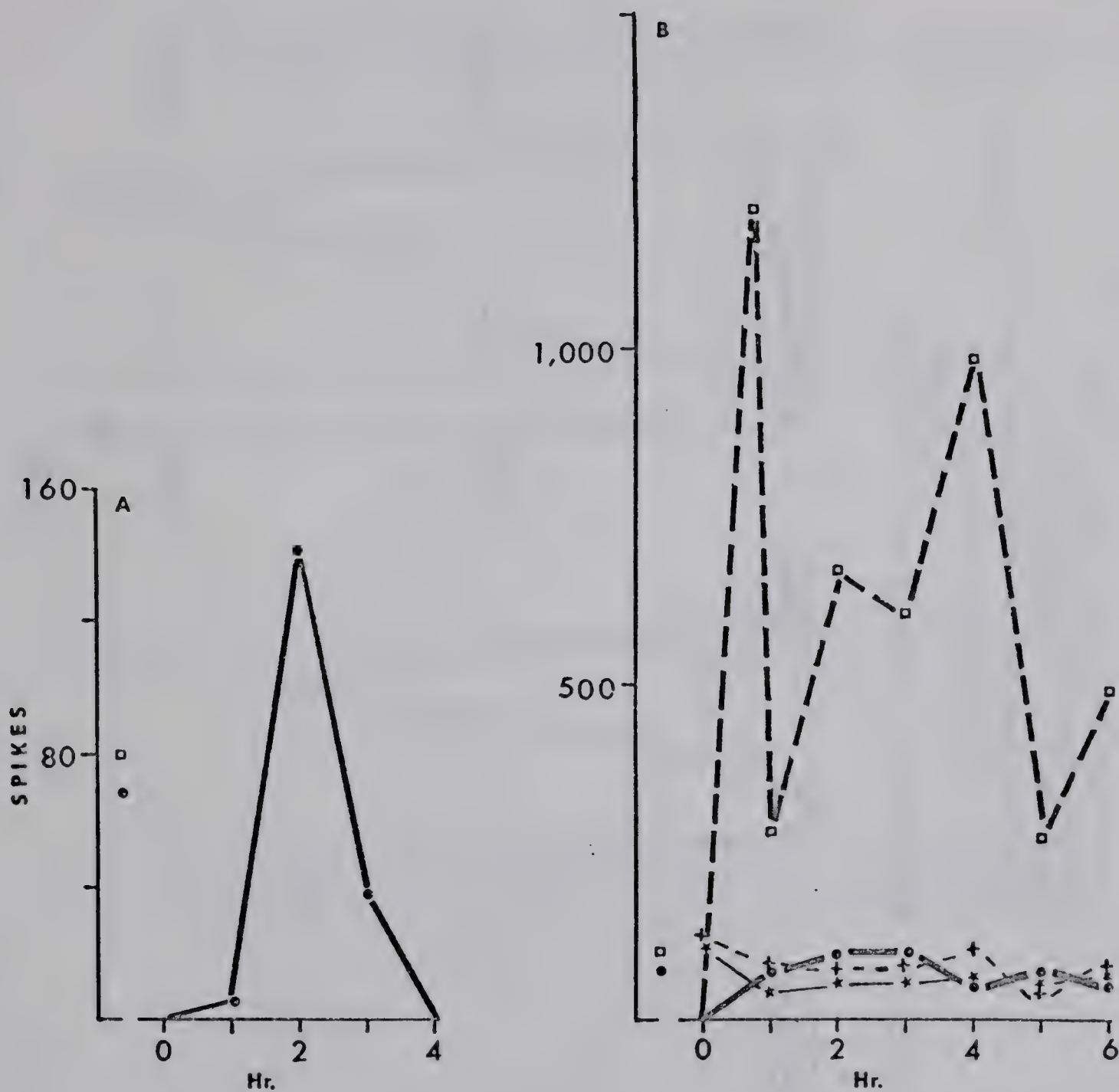
Fig. 22   Electrical activity of roaches treated with 10^{-4} M TEPP.

● spikes/sec.;   ▫ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.
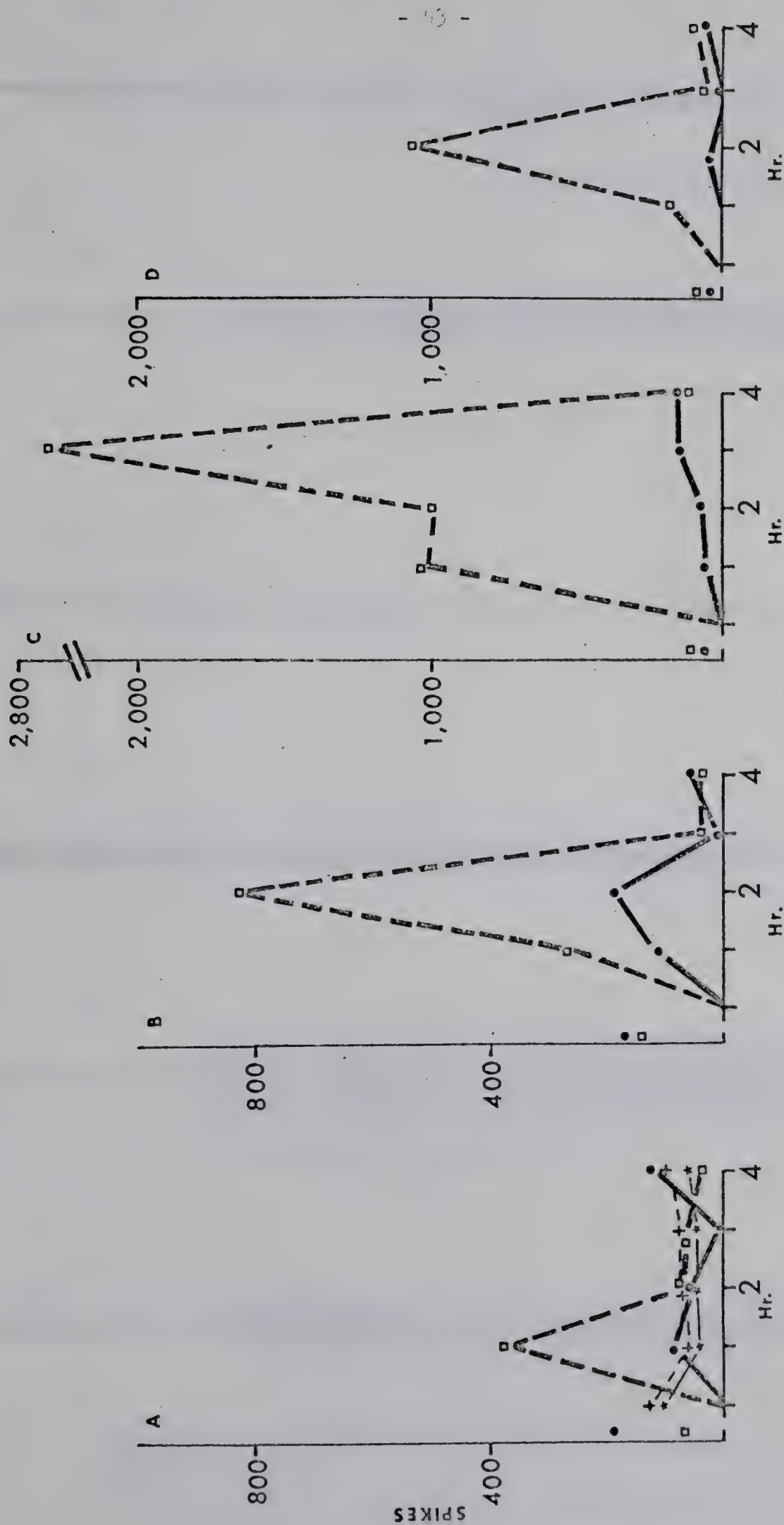
Fig. 23   Reactivation of electrical activity by 2-PAM.

A & B: 2 roaches treated with 10^{-3} M TEPP;
C & D: 2 roaches treated with 10^{-4} M TEPP;
10^{-3}M 2-PAM added at 0 hr. when complete block was observed.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.
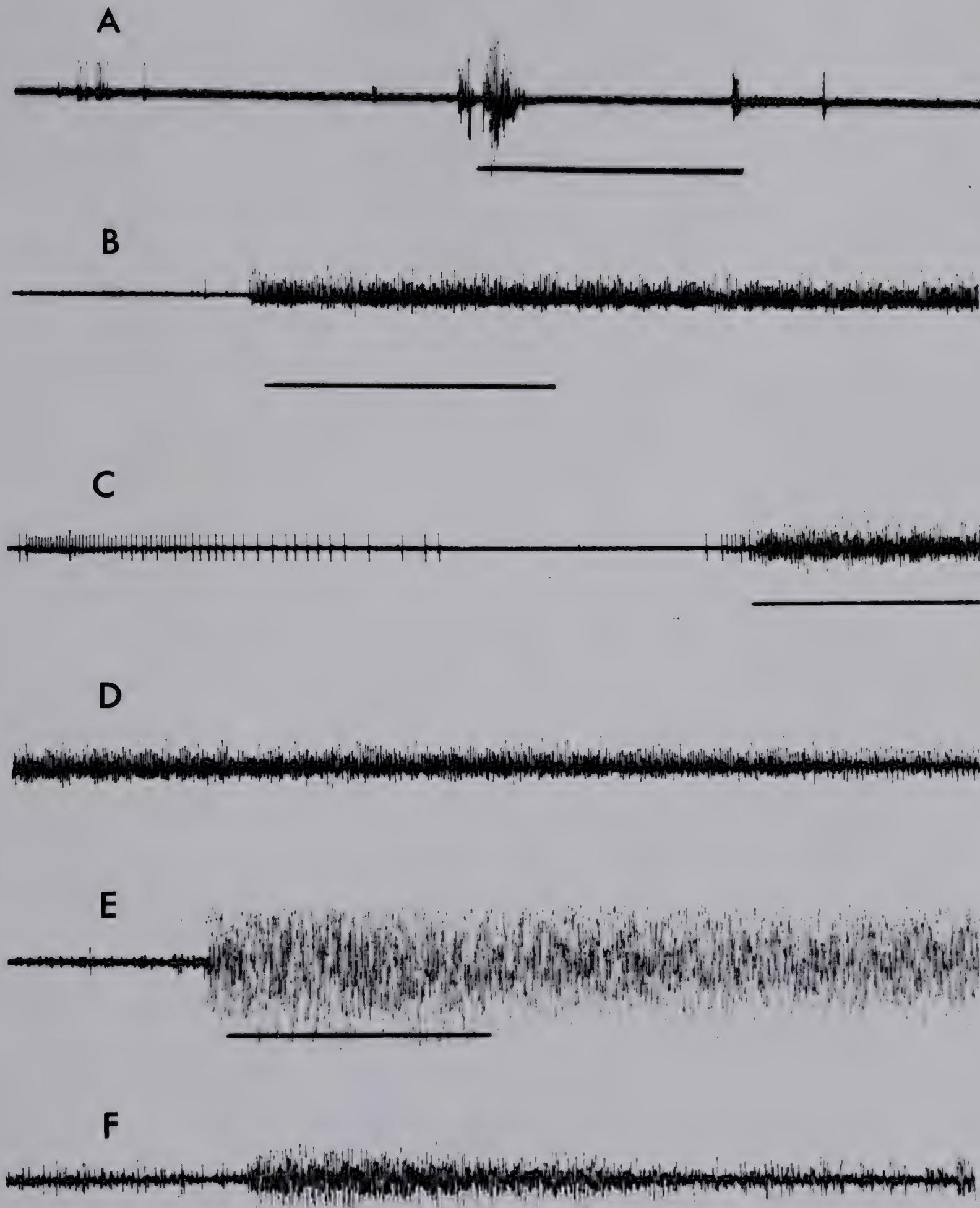
Fig. 24   Reactivation of electrical activity of a roach treated with 10^{-4} M
TEPP by 2-PAM.

(A) before the nerve cord was treated with TEPP. Electrical
activity ceased at 0 hr.

(B) 1 hr. after 10^{-3} M 2-PAM added;

(C) & (D) 2 hrs.;   (E) 3 hrs.;   (F) 4 hrs.

Solid line air puff response. Film speed 10 cm./sec.
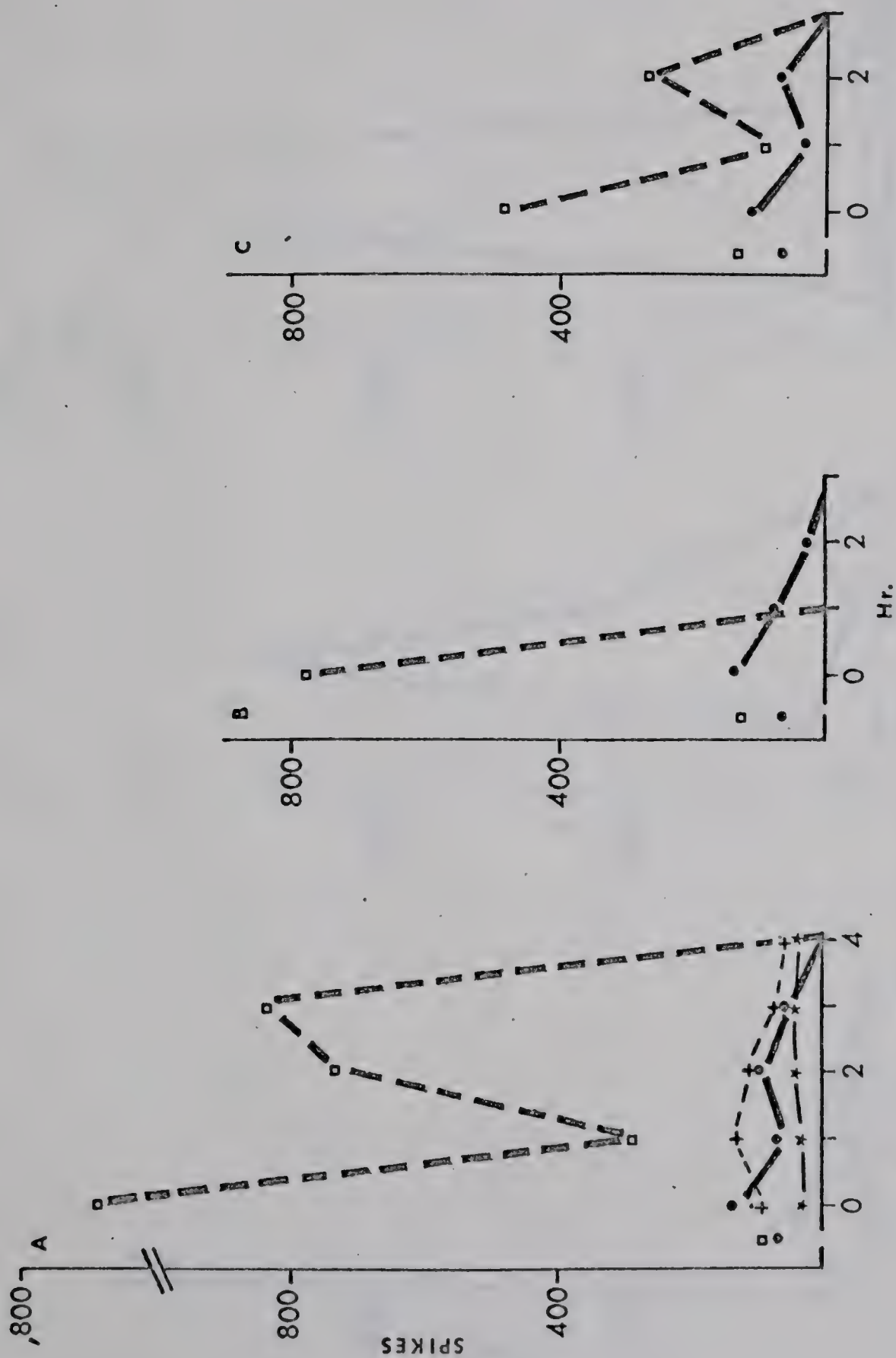Vertical scale 800 μv/1.2 cm.
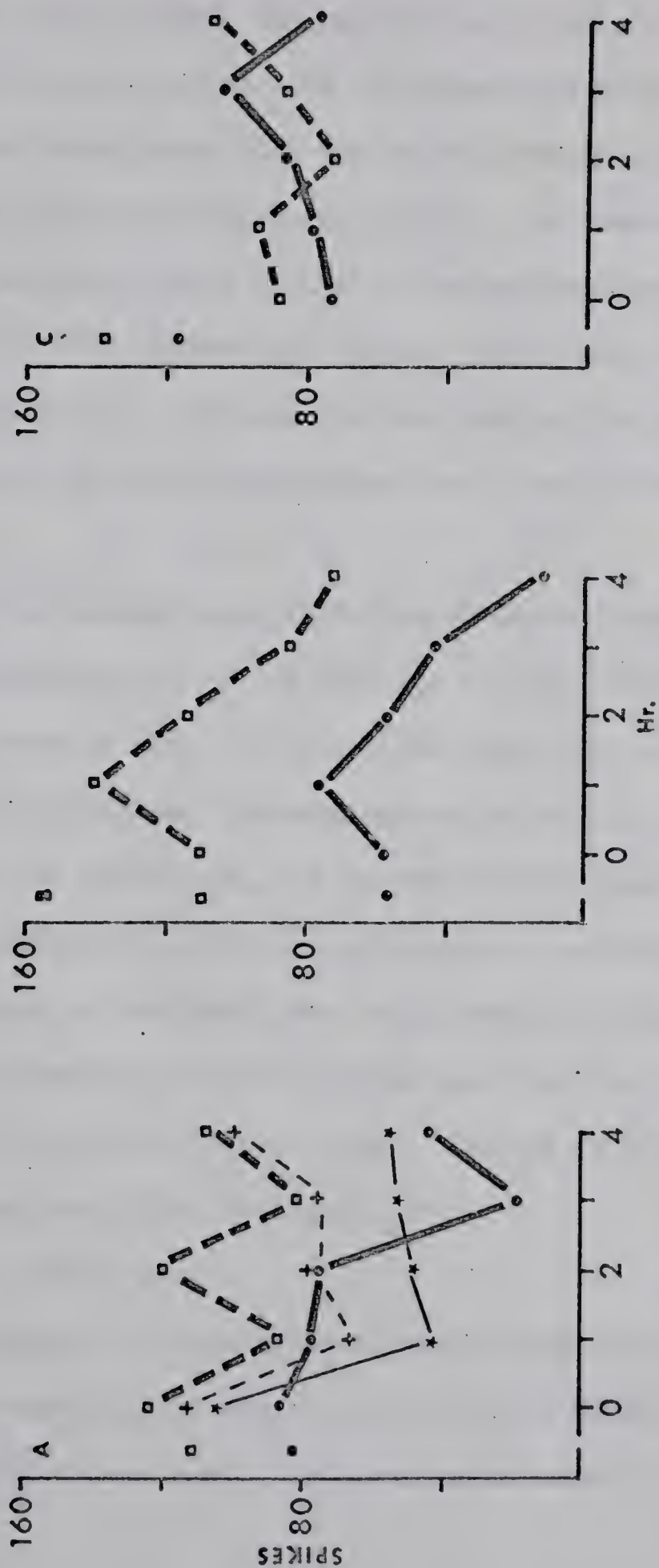
Fig. 25   Electrical activity of 3 roaches treated with 10^{-3} M 2-PAM.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
+ average spikes/air puff, saline control.

that the reactivation of phosphorylated AChE by 2-PAM involves the bonding of 2-PAM's quarternary ammonium group to the anionic site of the phosphorylated AChE, thus allowing the hydrolysis of the phosphorylated-oxime-enzyme complex to occur, giving AChE and phosphorylated-oxime.

Phosphorylated roach AChE was reactivated by 2-PAM in vitro (Colhoun, 1959b; Brady and Sternburg, 1966). The dose of 2-PAM necessary to reverse neuromuscular block in the rat nerve-diaphragm was close to the toxic dose for TEPP (Holmes and Robins, 1955), and the time taken for the reversal of block by 2-PAM was the same whether the anticholinesterase had been in contact with the preparation for a few minutes or for many hours.

5.23 The Effect of Choline upon TEPP-Treated Nerve Cords:

After immersion in 10^{-3} M TEPP for thirty minutes, three nerve cords were then treated with 10^{-3} M choline (Fig. 26). One preparation remained completely blocked. The endogenous activity of another preparation appeared at the third hour, but became blocked again at the fourth hour. Both the endogenous activity and synaptic transmission of a third preparation resumed at the third hour, with typical signs of AChE inhibition. But the endogenous activity stopped again an hour later, with synaptic block to follow two hours later. Choline is a weak reactivating agent of phosphorylated AChE (Hobbiger, 1963).

5.24 The Effect of Diazinon:

Since Diazinon is a phosphorothionate which must be converted to its oxidized analog, phosphate, to be active (O'Brien, 1960), a high concentration (10^{-2} M) was used. Antidromic discharge became apparent
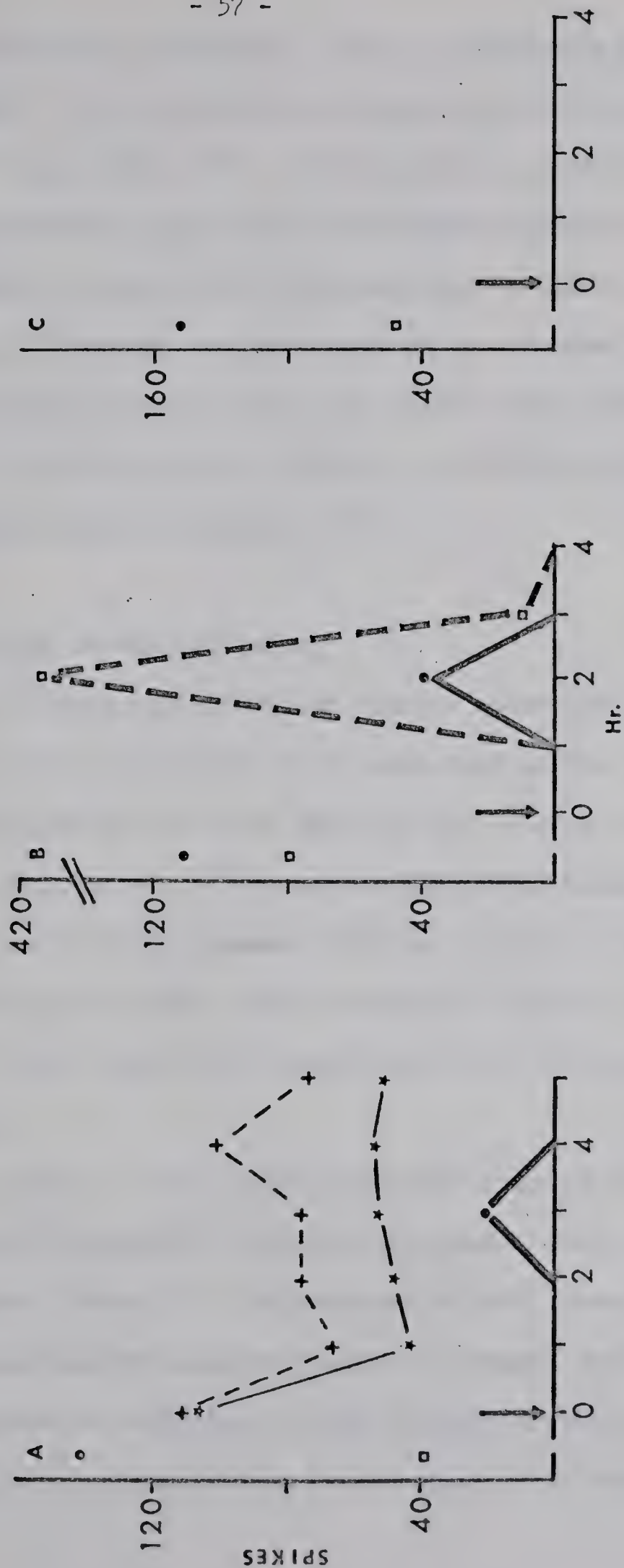
Fig. 26   Effect of 10^{-3} M choline on TEPP-treated roaches (10^{-3} M).

• spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
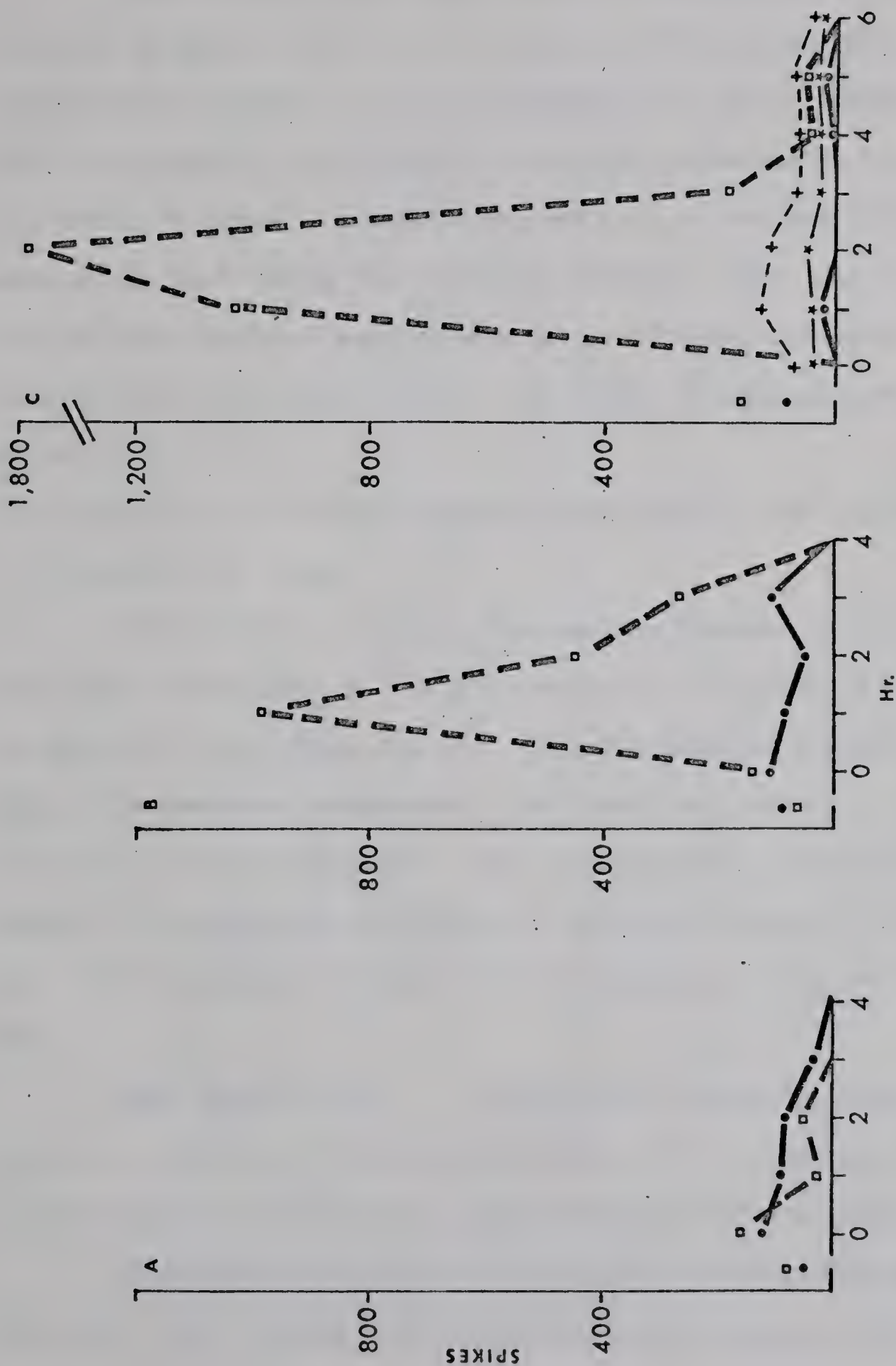+ average spikes/air puff, saline control;
↓ choline added.

one to two hours after application. But the observable effect was not as striking as TEPP. The time taken for complete electrical block to occur was 3, 5, and 8 hours (Fig. 27). The long latent period for the appearance of antidromic discharge may be due to the time necessary for the conversion of Diazinon into its active form (Yamasaki and Narahashi, 1960). Three to seven per cent of Diazinon was converted to the oxidized analog in the roaches (Kruger and O'Brien, 1960), one to two hours after injection. The nerve cords of roaches were most effective in metabolizing parathion to paraoxon (Chamberlain and Hoskins, 1951).

6.   Determination of AChE Activity:

Fig. 28 shows the effects of eserine concentration upon the rate of α-naphthylacetate hydrolysis by the esterases in the roach 6th abdominal ganglion. About 50% of the total activity was readily inhibited by 10^{-8} M eserine. The addition of 10^{-6} M eserine apparently blocked the AChE completely. This is in full agreement with the results of van Asperen (1962) when fly-head AChE was used. Using manometric technique, Chadwick (1947) reported that 96 per cent of the roach nerve cord AChE was inhibited by 10^{-5} M eserine.

The activity of the individual ganglia varied from 1.520 μM naphthol to 2.960 μM naphthol produced per minute, with a mean of 1.947 μM naphthol per min. (Table 1). The mean activity of three pooled homogenates was 2.111 μM naphthol produced per minute. Yamasaki and Narahashi (1960) observed considerable variation of AChE activity in roach nerve cords.

After an electrical block became apparent in three roach nerve

Fig. 27 Electrical activity of 3 roaches treated with 10^{-2} M Diazinon.

- spikes/sec.;   □ spikes/air puff;
- average spikes/sec., oil control;
- average spikes/sec., oil control;
+ average spikes/air puff, oil control.
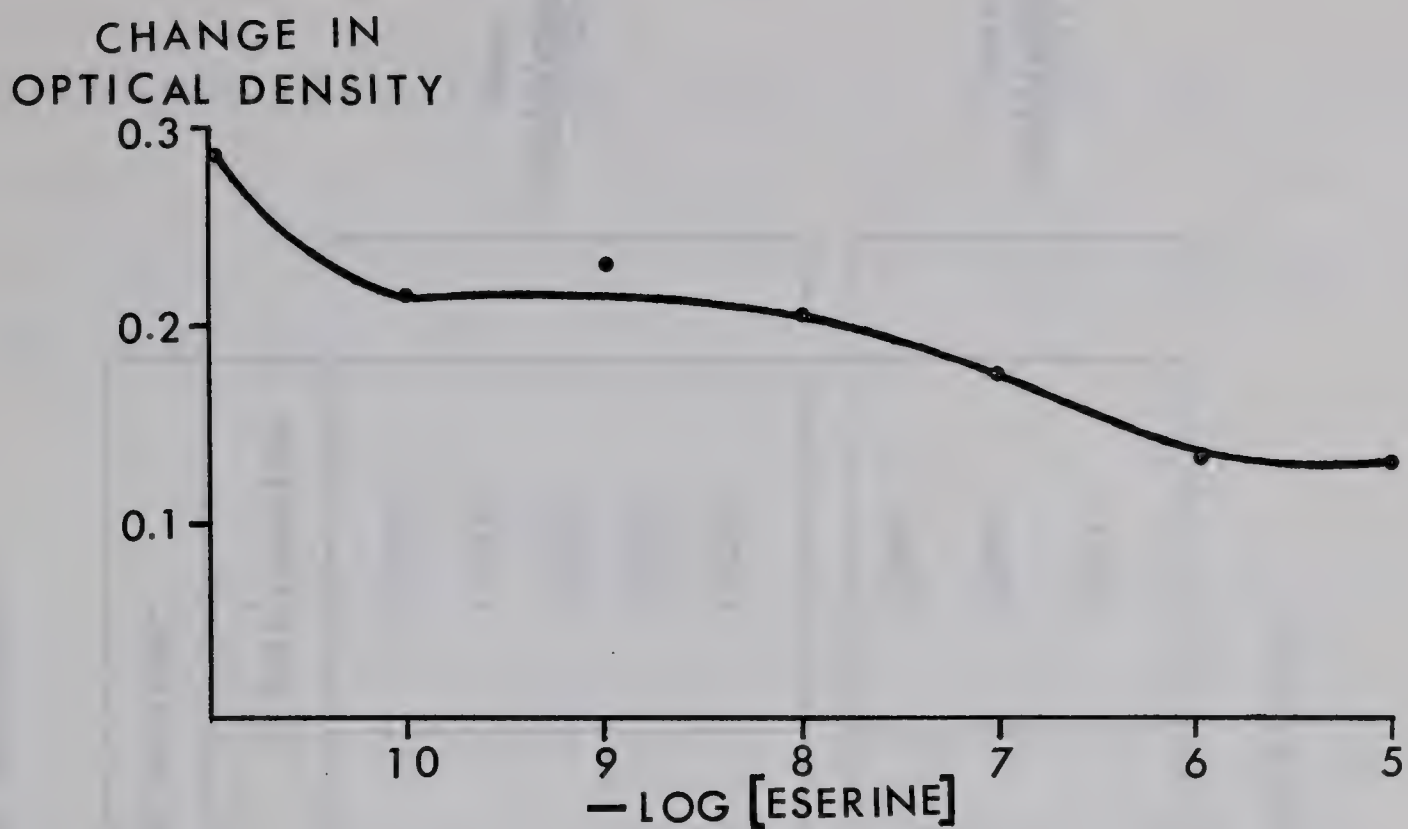
Fig. 28   Inhibition of roach ganglion AChE activity by eserine.
Temp. 40°C.   Substrate 3×10^{-4} M α-naphthyl acetate.
Homogenate concentration 1/4 ganglion per tube.
Incubation time 10 min.
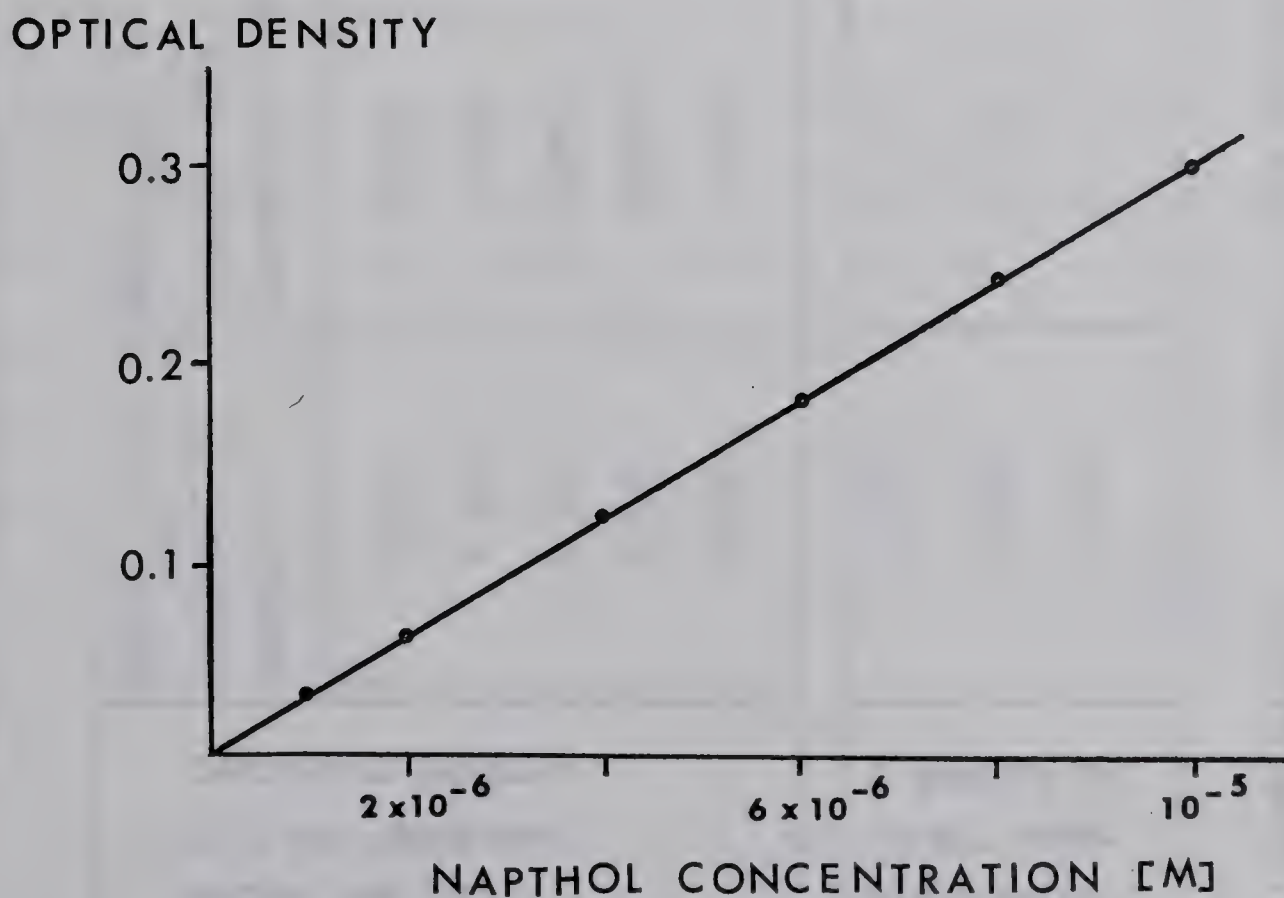
Fig. 29   Naphthol concentration and corresponding optical
density.

Table 1: AChE activity in 6th abdominal ganglion of P. americana.

	EΔ$O.D._{600}$/0.25 ganglion less eserine	IΔ$O.D._{600}$/0.25 ganglion with eserine (10^{-5} M)	AChE Activity*	
			NΔ$O.D._{600}$/0.25 ganglion	Unit[1]/ganglion
Individual ganglion in 2 ml. phosphate buffer (pH 7.0)	0.415	0.296	0.119	1.587
	0.320	0.193	0.127	1.693
	0.445	0.297	0.148	1.973
	0.488	0.266	0.222	2.960
	0.268	0.154	0.114	1.520
5 ganglia in 10 ml. phosphate buffer	0.293	0.120	0.173	2.307
	0.293	0.158	0.135	1.800
	0.278	0.111	0.167	2.227

Mean unit/ganglion 1.947

Mean unit/ganglion 2.111

* AChE activity = EΔ$O.D._{600}$ less eserine – IΔ$O.D._{600}$ with eserine

[1] One unit activity = 1 μM. of naphthol produced/min.

$O.D._{600}$ 0.03 = 1 μM.

cords treated with 10^{-4} M TEPP, the AChE activity was assayed immediately (Table 2). The mean AChE activity was 11.162% of that of the control. Inactivation of AChE ran parallel with the synaptic after-discharge (Yamasaki and Narahashi, 1960).

7.   The Effect of Adrenergic Drugs:

Phenoxybenzamine (dibenzyline) blocks the α-receptors of the adrenergic nerves effectively and persistently (Nickerson, 1965). When phenoxybenzamine (10^{-3} M) was applied to the nerve cords of roaches, a blocking effect was observed (Fig. 30). Washing with saline did not eliminate the blocking effect. Being a β-haloalkyl agent that can cause tissue damage as nitrogen mustard (Nickerson, 1949), phenoxybenzamine may not be specific in its action.

Monoamine oxidase (MAO) is the enzyme chiefly responsible for the physiological inactivation of 5-hydroxytryptamine (5-HT), and endogenous catecholamines (Kopine, 1964; Page, 1958; Shore et al., 1957). Tranylcypromine, a MAO inhibitor, blocked the endogenous activity and the synaptic transmission of the nerve cords of roaches at 10^{-3} M (Fig. 31). MAO inhibitors can produce an irreversible inactivation of MAO by forming stable complex with the enzyme, causing an elevation of biogenic amines (Javik, 1965; Pletscher, 1966). But MAO inhibitors can also inhibit other enzymes as well (Javik, 1965).

8. Fluorometric Determination of Catecholamines:

There was no indication of the presence of either dopamine or

Table 2: AChE activity in 6th <u>abdominal</u> ganglion of
P. americana treated with 10^{-4} M TEPP.

Individual ganglion in 2 ml. phosphate buffer (pH 7) containing 0.3% ACh	EΔO.D.$_{600}$/0.25 ganglion less eserine	IΔO.D.$_{600}$/0.25 ganglion with eserine (10^{-5} M)	AChE Activity*		
			NΔO.D.$_{600}$/0.25 ganglion	Unit[1]/ganglion	% control[+]
	0.037	0.016	0.021	0.280	14.380
	0.021	0.001	0.020	0.266	13.662
	0.018	0.010	0.008	0.106	5.444

* AChE activity = EΔO.D.$_{600}$ less eserine - IΔO.D.$_{600}$ with eserine

[1] One unit activity = 1 μM of naphthol produced/min.

O.D.$_{600}$ 0.03 = 1 μM

Mean unit activity = 11.162% of control

[+] Control = Mean AChE activity of individual ganglia in Table 1
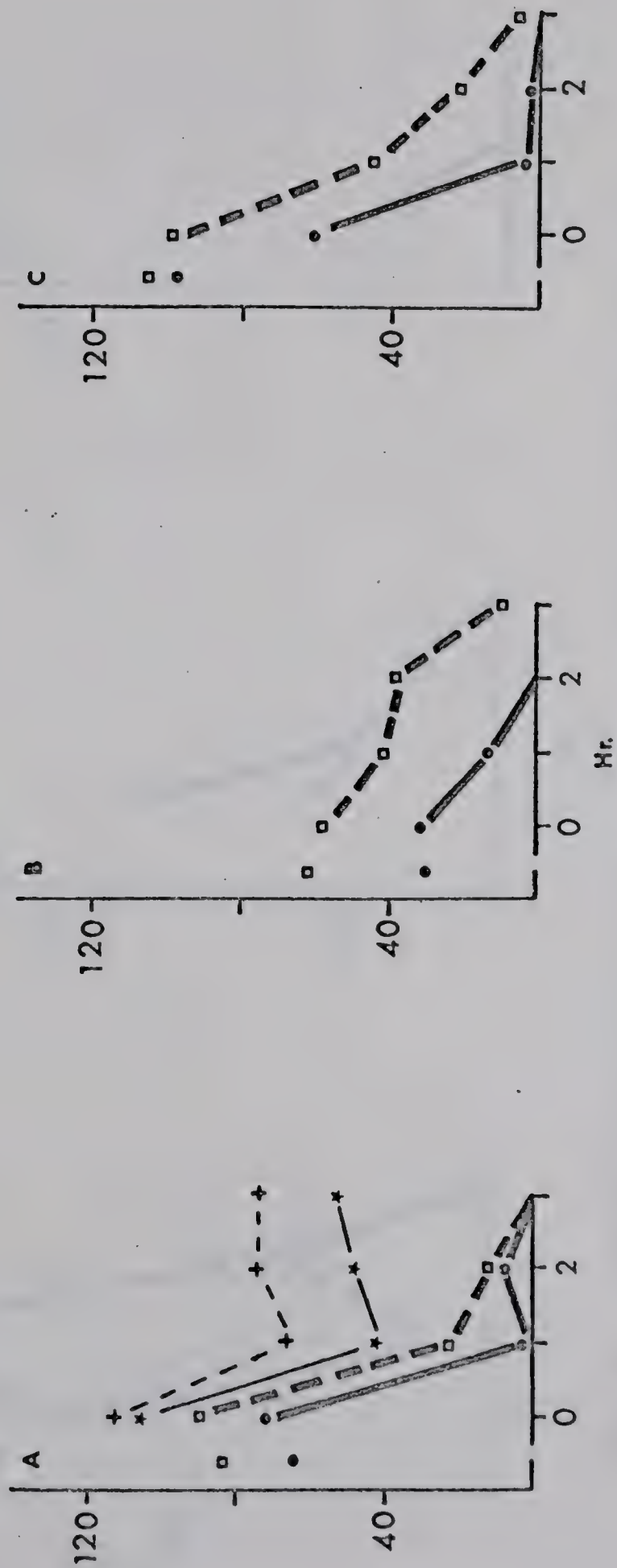
Fig. 30   Electrical activity of 3 roaches treated with 10^{-3} M phenoxybenzamine.

● spikes/sec.;   □ spikes/air puff;
★ average spikes/sec., saline control;
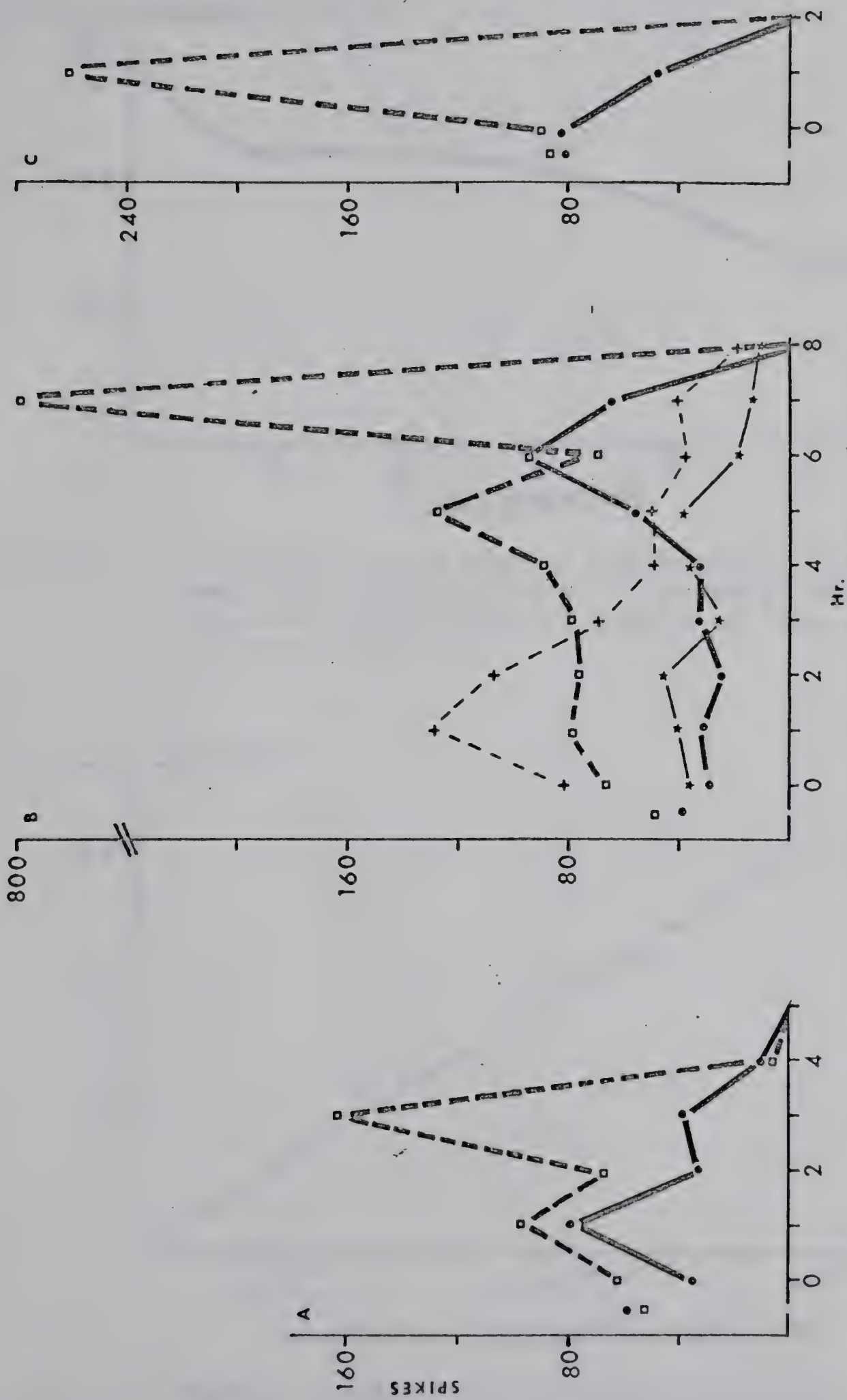+ average spikes/air puff, saline control.
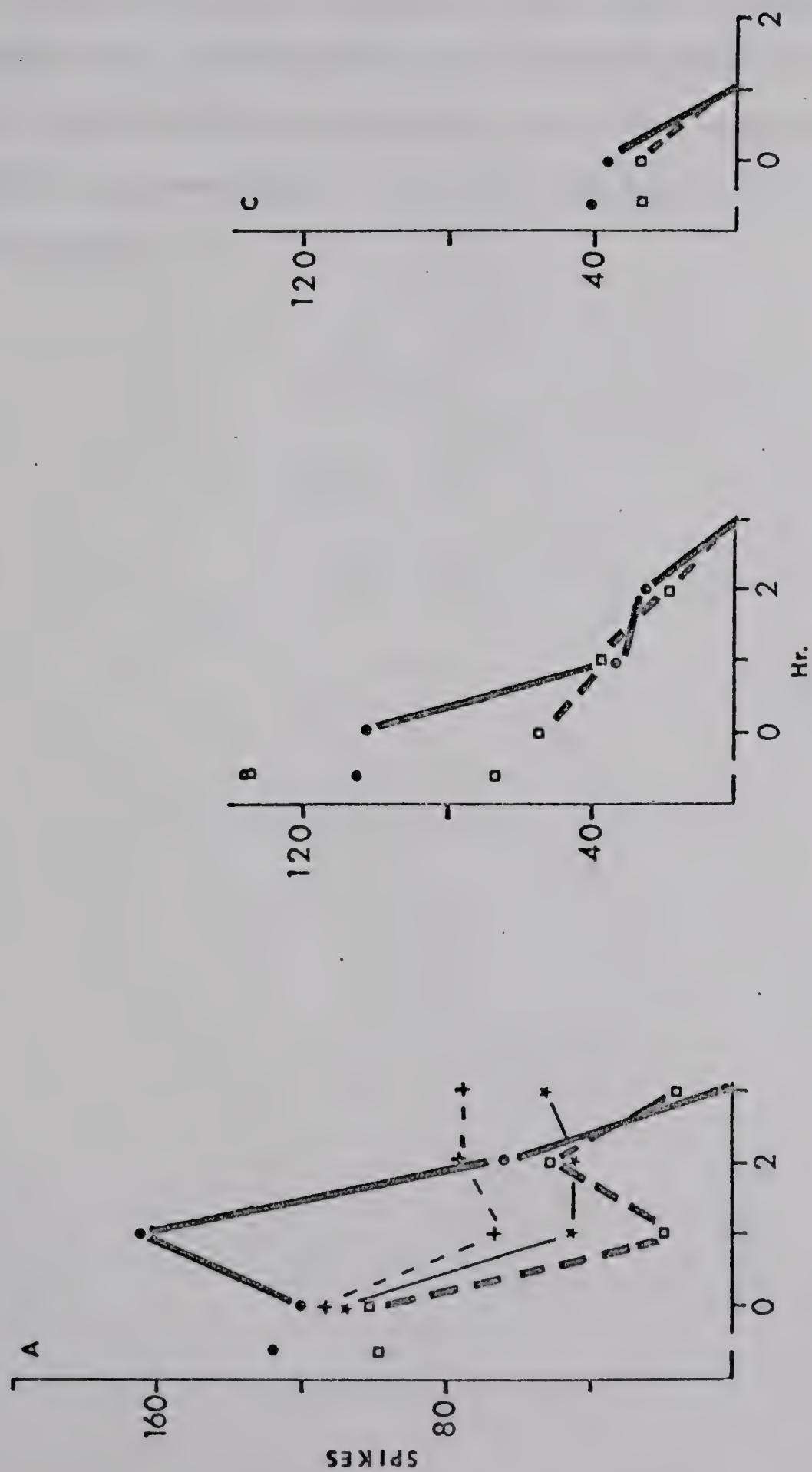
Fig. 31   Electrical activity of 3 roaches treated with 10^{-3} M tranylcypromine.

• spikes/sec.;   ▫ spikes/air puff;
★ average spikes/sec., saline control
+ average spikes/air puff, saline control.

noradrenaline in the sample (Table 3; Fig. 32). Owing to the large

number of roaches required to yield sufficient amount of nerve cords,

the experiment was run only once. Unger (1957) believed that adrenaline,

noradrenaline, and histamine are not among a number of cardiac acceler-

ators obtained from the abdominal nerve cords, corpus allata, corpus

cardiaca and haemolymph. Fluorescence microscopy will bring light to

such subject.

Table 3:   The fluorescence O.D. of standard dopamine and
extraction sample of roach abdominal nerve cords;
excitation wavelength at 345 mμ;   fluorescence
wavelength at 410 mμ.

Material	Fluorescence O.D.
Std. dopamine$_1$ (0.25 μg.)	1.08
Std. dopamine$_2$ (0.25 μg.)	1.10
Sample$_1$	0.28
Sample$_2$	0.28
Tissue blank	0.28
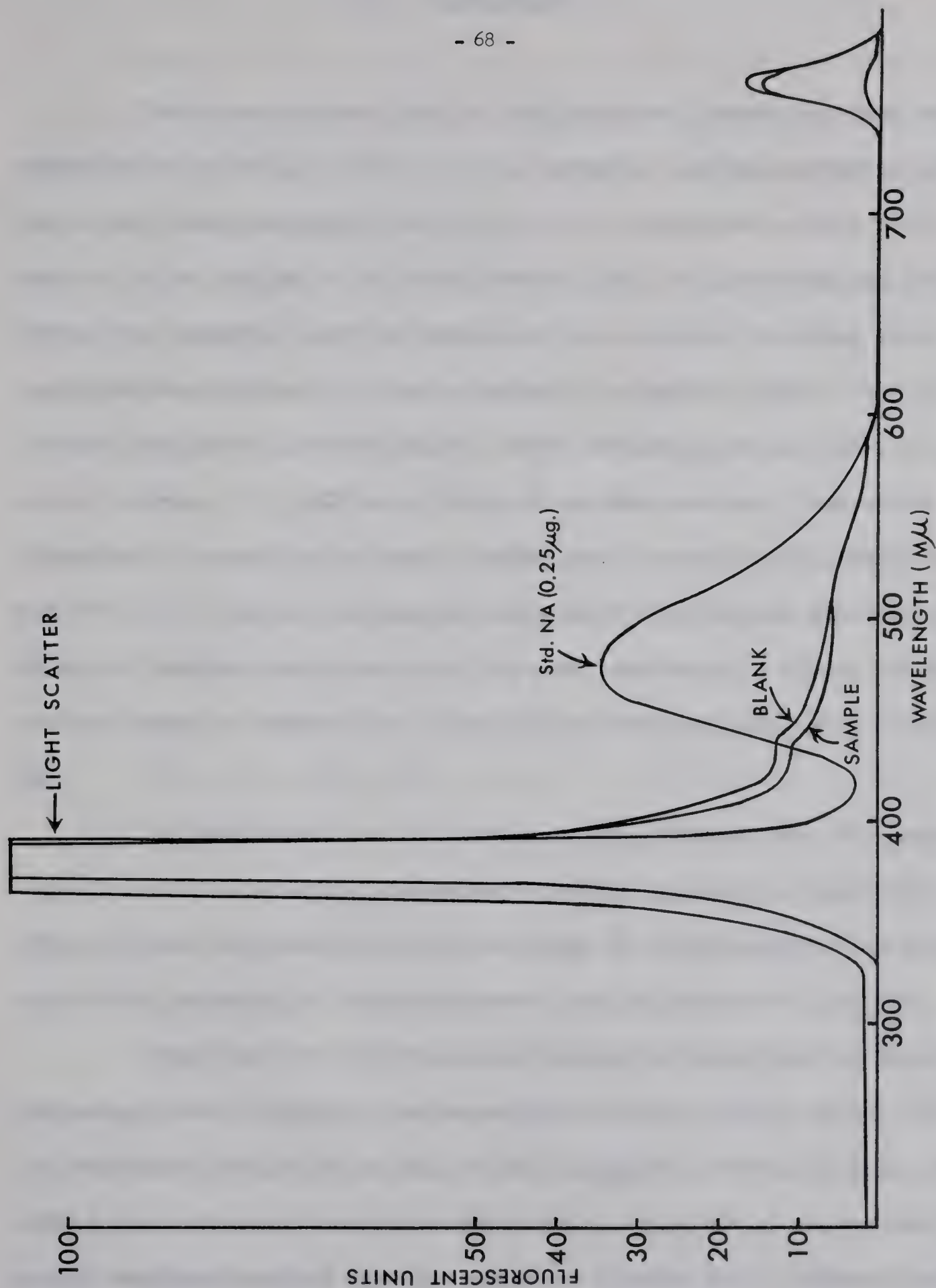
Fig. 32   Fluorescence spectra and light scatter of 0.25 μg of standard noradrenaline, a sample of extraction of roach abdominal nerve cords, and a reagent blank assayed by the iodine method. Excitation was at 385 mμ.

IV. DISCUSSION

The physiological role of ACh in nerve tissues has been reviewed extensively by Koelle (1963). In the synaptic and neuroeffector sites, there are three possible roles that can be attributed to ACh: (1) On arrival of an impulse in an axon terminal, ACh is liberated and diffuses across the synaptic cleft to combine with a receptor to bring about a localized depolarization, the postsynaptic potential (PSP). The latter in turn initiates electronically a nerve action potential (NAP) in the second neuron. (2) ACh acts first at an axon terminal from which it is liberated to cause the release of additional quanta of ACh, which produce the PSP. (3) In non-cholinergic neurons, NAP liberates ACh from pre-synaptic terminals, which acts at the same terminals to effect release of another synaptic transmitter. The latter produces PSP, which initiates NAP.

The demonstration that hemicholinium blocked both the endogenous activity and the synaptic transmission either entirely or partially (Fig. 4), and the reactivation of activity by choline,indicates that the electrical activity of the roach nerve cord is dependent upon ACh.

Drugs that act at cholinergic sites are generally divided into two categories: nicotinic and muscarinic (Albert, 1965; Goth, 1966). The muscarinic drugs act mainly on the peripheral nervous system, stimulating the post-ganglionic parasympathetic receptors of organs such as smooth muscles, cardiac muscle, endocrine glands, etc., without having an effect upon ganglionic transmission or skeletal muscles (Goth, 1966). The

nicotinic drugs can stimulate autonomic ganglion and end-plates of skeletal muscles (Goth, 1966). The nicotinic receptors are divided into the ganglionic receptors which are hexamethonium sensitive, and the skeletal muscle receptors which are sensitive to curare (Goth, 1966). Nicotine has effects on both the ganglionic and neuromuscular transmission (Albert, 1965; Goth, 1966). However, these are only working hypotheses for mammalian peripheral nervous system. The terms "nicotinic" and "muscarinic" are not applicable to the central nervous system (Curtis, Ryall and Watkins, 1965; Feldberg, 1950).

Klromov-Borisov and Michelson (1966) pointed out that the invertebrate muscles are mainly nicotinic. This invites the speculation that the roach cercal synapse does not have a muscarinic receptor. The failure of atropine and methacholine to produce any blocking or killing effect (Roeder, 1948b; Tobias et al., 1946) supports such a hypothesis.

Ambache (1955) pointed out the limitations of using atropine as a pharmacological criterion for cholinergic nerves: (1) atropine might be destroyed by an esterase; (2) true atropine resistance in cholinergic systems might be due to "proximity"; (3) secondary formation of atropine resistant pharmacological agents.

The failure of DMPP, a nicotinic drug, to produce any observable effects points to the uniqueness of the roach cercal ganglion.

It is of interest to compare the in vivo effects of injecting carbachol, ACh, and methacholine into roaches with the results of the present investigation. The toxic dose of carbachol for the roaches was 0.5 to 0.1 g./kg., 7 to 10 g./kg. for the ACh; methacholine even at

20 g./kg. produced no killing effect (Tobias et al., 1946).

Treherne (1966) postulated a "biochemical barrier" theory, which is partly based on the histochemical evidence that AChE is situated "strategically" on the glial membranes bordering extracellular channels. Despite the rapid influx of ACh, the concentration of ACh within the roach ganglion was reduced to 8.1×10^{-5} M when the nerve cord was bathed with a solution of 10^{-2} M ACh (Treherne and Smith, 1965b). The relative inability of externally applied ACh to affect insect nerves would thus seem to result from the presence of this "biochemical barrier" rather than to the "ion barrier" (Treherne, 1966).

Although choline could block synaptic transmission, the overall effect of the ACh in the present investigation could not be explained entirely by the hydrolysis of ACh to choline and acetate. In addition to its depolarizing property, it is possible that ACh also hyperpolarizes or inhibits nerve cells in the roach cercal ganglion. When the nerve cords were isolated, or desheathed, ACh was allowed to reach a cholinoreceptive site to cause depolarization. This would be completely in accord with the finding that there are multiple cholinoreceptive sites within the mammalian ganglia, and that ACh can be excitatory or inhibitory depending on the receptor (Eccles and Libet, 1961; Geber and Volle, 1965; Koelle, 1965; Takeshige and Volle, 1963, 1964). Since the ganglionic potentials represent an algebraic summation of simultaneous processes, it is conceivable that the temporally related hyperpolarization and postganglionic firing resulted from monitoring certain populations of cells and fibers (Takeshige and Volle, 1964). Thus, while the ganglion as a whole appeared

to be in a hyperpolarized state, the postganglionic fibers monitored might
have been partially depolarized (Takeshige and Volle, 1964). Unfortunately,
this possibility could not be tested with the present experimental tech-
nique. The results of carbachol, pilocarpine and Tremorine could also be
explained by this postulation. It is also possible that ganglionic hyper-
polarization caused by ACh was mediated by means of an inhibitory trans-
mitter liberated within a ganglion (Volle, 1965).

It was recently found that ACh acts both as an excitatory and
inhibitory transmitter in the same abdominal ganglion of two species of
marine mollusc, Aplysia (Kandel and Frazier, 1967; Tauc and Gerschenfeld,
1962). Two types of ganglion cells have been demonstrated by the actions
of ACh. The response of one group of cells to ACh was characterized by
depolarization and acceleration of the rate of firing. Conversely, ACh
produced hyperpolarization and the blockade in the second group.

The possible role of ACh may play in the sodium pump mechanism
(Hokin, Hokin and Shelp, 1960) should not be overlooked. A given drug,
such as ACh itself, may produce either cholinomimetic or cholinergic
blocking action at certain sites, depending upon the dose, rate of com-
bination with the receptors, and other factors (Koelle, 1962; Paton,
1961). Also of equal importance is the role the inhibitory system may
play when the insect CNS is either intact or semi-intact (Milburn, Weiant,
and Roeder, 1960; Roeder, 1962).

Treating the roach with DDT resulted in a marked accumulation
of ACh although the AChE was not inhibited (Colhoun, 1959a,b; Tobias
et al., 1946). A neuroactive substance was released into the blood of

the roach during the course of DDT poisoning (Colhoun, 1959b; Sternburg et al., 1959). It was also suggested that the source of the toxicant was apparently the CNS itself during periods of great nervous activity, whether initiated by electrical stimulation or by constant bombardment of sensory-central synapses due to excessive afferent impulses generated in the sensory nerves by direct action of DDT (Sternburg et al., 1959).

Koelle (1963) surmised that the earliest function of ACh and its associated enzymes in primitive organisms was probably the modification of the passage of various substances across the cell membranes. With the subsequent development of different types of structural complexity of cellular membranes in accordance with their various functions, the ACh-AChE system itself has been achieved in nervous tissues, where its components and function are concentrated predominantly at synapses and junctional sites. At such regions, the ACh may serve both as the transmitter and as the agent for the liberation of other chemical mediators.

Catecholamines have been demonstrated by fluorescence microscopy in the autonomic ganglia or mammals (Jacobowitz and Koelle, 1963; Hamberger, Norberg and Sjöqvist, 1965; Hamberger, Norberg and Unguestedt, 1965; Norberg and Sjöqvist, 1966; Owen and Falk, 1965). Noradrenaline and adrenaline have both inhibitory and facilitatory actions on sympathetic ganglia of the cat (de Groat and Volle, 1966a,b). Eccles and Libet (1961) proposed that ACh can release an adrenergic substance through a chromaffin cell to produce an action potential. It was postulated that ACh released from sympathetic fibers could liberate noradrenaline

from a peripheral store, in the same way as injected ACh (Burn, 1966;
Burn and Rand, 1965; Chang and Rand, 1960).

The possibility that catecholamines may participate in roach
synaptic transmission was suggested by Twarog and Roeder (1957), who
recorded asynchronous bursts of low voltage spikes following application
of adrenaline and noradrenaline to desheathed roach ganglion. After-
discharge and synaptic blocking were observed at concentrations between
10^{-3}M and 10^{-2} M. But when applied to intact ganglion, noradrenaline
and adrenaline failed to show any stimulatory or blocking effect
(Colhoun, 1959a). Application of 5×10^{-5} M dopamine to the roach
cercal ganglion induced bursts of activity which propagated along the
nerve cord; however, this substance did not have any effect on the
synaptic transmission (Gahery and Boistel, 1965). Since not all the
regions in the roach cercal ganglion, as shown by electron micrograph,
are associated with membrane-bound cholinesterase activity (Smith and
Treherne, 1965), such non-cholinergic synapses could be regarded as
possible candidates for transmission mechanisms involving catecholamines
(Treherne, 1966).

Injection of adrenaline into roaches paralyzed them temporarily,
but injection of noradrenaline produced no recognizable response (Barton
Brown et al., 1961). All adrenergic neuron blocking agents examined in
detail shows a variety of actions at cholinergic sites (Boura and Green,
1965).

Colhoun (1963a) showed the presence of 5-HT in the nerve cords
of roaches, though the amount is extremely low in comparison with ACh.

Grollman (1960) believed that 5-HT is a primitive synaptic transmitter. It is a chemical transmitter in some invertebrates and vertebrates (Brodie and Shore, 1957; Owen and Falck, 1965; Welsh, 1957), and can potentiate and buffer ganglionic transmission (Douglas, 1965; Page, 1958). If some of the non-cholinergic regions are occupied by 5-HT, then the data for the adrenergic drugs in the present investigation become interpretable.

Catecholamines and adrenaline-like substances have been extracted from insects (Cameron, 1953; Gregerman and Wald, 1952; Ostlund, 1954; von Euler, 1961). The neurosecretion from corpus cardiacum of P. americana and Rhodnius sp. is related to adrenaline (Barton Browne et al., 1961; Wigglesworth, 1954). The present investigation conforms to the belief of Unger (1957) that catecholamines are not present in the nervous tissue of roaches.

Four possible functions have been proposed for the AChE in cholinergic nervous systems (Koelle, 1963; Volle and Koelle, 1961): (1) temporal or spatial limitation of the transmitter action of ACh at the postsynaptic site; (2) rapid hydrolysis of ACh to provide an immediate source of choline for uptake by the presynaptic terminals and synthesis to ACh; (3) protection of the presynaptic terminals against reactivation by self-liberated ACh; (4) prevention of accumulation of activating concentrations of ACh during the resting stage.

Confirmation of the first and fourth proposal can be obtained by the results of anticholinesterases (Figs. 20, 21, 22, 27; and Table 2), only on the ground that ACh is a transmitter that diffuses across the

synaptic cleft to act on a postsynaptic receptor site in the roach cercal ganglion. The synaptic after-discharge, the antidromic firing, and electrical quiescence were probably due to the accumulation of endogenous ACh on the postsynaptic sites. It is also possible that ACh caused another transmitter substance to be released and act on the postsynaptic sites, and since the ACh was uanble to be hydrolyzed, it blocked all the sites that would release the stimulatory transmitter.

No evidence was obtained to support the second proposal. When choline was applied to the nerve cords with or without AChE-inactivation by TEPP, it either could not restore the electrical activity to its normal state or it only accelerated the rate of failure of postganglionic firing. Nothing can be said about the third proposal until more is known about the synapses in insects.

The present investigation brought out the effectiveness of the "biochemical barrier." Indeed, such a barrier may be highly advantageous for the insects, because of their open circulatory system. Ginetsinskii (1947) concluded that in the course of evolution, reduction occurs in the cholinoreceptive zone, and in the number of drugs to which the cholino- receptive zone is sensitive, i.e., an increase in the precision and specificity of cholinoreception. Such may be the case for the insects. The "biochemical barrier" and the cholinoreceptive specificity may explain the well protected synapses in insect ganglion.

The experiments with 2-PAM (Fig. 23) point to the prime importance of AChE in synaptic transmission, although 2-PAM was also shown to reactivate axonal transmission (Dettbarn, Rosenberg and Nachmanson, 1964).

The concentration of AChE in a given neuron reflects the extent of the participation of ACh in the synaptic transmission (Koelle, 1962).

The slow in vivo inhibition of AChE by the carbamates indicates the effective concentrations of ACh at the active sites of enzymes are normally very low (Winteringham, 1966). Stimulated central activity may, accordingly, result in relatively large increases in effective substrate concentrations at the enzyme sites, but this would not be reflected in detectable changes in total ACh content of the central nervous system, and may only involve a small fraction of the total ACh (Winteringham, 1966).

Smallman and Fisher (1958) observed an increase of ACh content in roach thoracic nerve cords treated with TEPP. Sublethal doses of TEPP resulted in transitory depression of AChE activity coinciding with a limited elevation of ACh levels followed by a return to normal; lethal doses result in prolonged inactivation of AChE and a corresponding steady increase of ACh to 90% above the normal level. Subsequent partial recovery of AChE activity, observed with lethal doses of TEPP and mal-athion, coincided with a fall in ACh values to below normal. Brady and Sternburg (1966) suggested that the recovery of AChE levels was a result of AChE synthesis and not a reversal of inhibited AChE.

The considerable variations of AChE concentration in the roach ganglion (Table 1) may explain the occasional ineffectiveness of ACh and some other related drugs at the same dosage. But that the electrical activity of one roach nerve cord could continue in the presence of high concentration of TEPP was perplexing, unless there is an enzyme in the

nerve cord that can hydrolyze TEPP. It was demonstrated that diisopropyl fluorophosphate (DFP) was hydrolyzed readily by a phosphatase to diisopropyl phosphoric acid inside a squid axon (Hoskin, Rosenberg and Brazin, 1966).

Since the whole field of insect neuropharmacology, as Colhoun put it, is "conditioned" by rather well documented experiments with mammals (E. H. Colhoun, personal communication), there are limitations in interpreting these data.

V. SUMMARY OF CONCLUSIONS

(1) Insect synaptic transmission apparently depends upon ACh. This is
     supported by the demonstration that hemicholinium blocked both
     the endogenous activity and synaptic transmission either entirely
     or partially (Fig. 4), and by the restoration of this activity by
     choline (Fig. 6).

(2) ACh is either a transmitter substance of a "trigger" substance
     that liberates other transmitter substances at the synapse of the
     6th abdominal ganglion of the roach. The high concentration of
     ACh and AChE at the synapse suggests that ACh is probably the main
     transmitter substance.

(3) AChE is vital for the normal synaptic function. This conclusion
     is based on the experiments with 2-PAM (Fig. 23), which reactivated
     synaptic transmission in TEPP-treated nerve cords, and the effects
     of anticholinesterases upon nerve transmission.

(4) The concentration of AChE in the 6th abdominal ganglion varied from
     roach to roach (Table 1).

(5) The fact that DMPP failed to produce any observable effect on the
     electrical activity of the roach nerve cords points to the differ-
     ence between an insect synapse and a mammalian autonomic synapse.

(6) Noradrenaline and dopamine could not be demonstrated in the abdominal
     nerve cords of the roach.

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